THE GROWTH OF THE KIDNEYS AND THE BLADDER IN THE FETAL DOG

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FOUR FIGURES

The qualitative development and structure of the kidneys are well described in the literature but there have been very few studies devoted to the quantitative development of the kidneys. The postnatal growth in weight of the kidneys are given for man by Peter ('27) and Scammon ('33) and Wald ('37), gives data on the weights of the adult kidneys. There is very little material on the prenatal growth of the kidney in man or in animals. The growth of the kidney in the fetal cat has been studied by Latimer ('39). This paper gives the references to some of the literature on the growth of the kidneys in man and in other animals, and this literature will not be repeated here. The only data found on the weights of the kidneys in the dog are for three fetal and one newborn dog (Liesenfield et al., '27), and the adult dog by Junkersdorf ('25) and Kunkel ('30).

There is even less quantitative data on the urinary bladder. It was not weighed in the fetal cats and data are available only for the adult cats. The capacity of the human bladder is discussed but no weights are available except for the newborn.

MATERIALS AND METHODS

The methods of collecting, preserving and dissecting the 156 fetal and 24 newborn dogs have been described in the preceding papers in this series (Corder and Latimer, '49; Latimer and Corder, '48; and Latimer '49 and '50) and need

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not be repeated here. Forty additional specimens have been added to the number since the first papers were published.

The two kidneys were removed, freed of fat, and the vessels and the ureters cut close to the hilum. They were kept in the moist container until weighed. The bladders likewise were removed, freed of fat and connective tissue and the ureters and urethra cut close to the viscus. Any urine found in the bladder was emptied and they were then put in the moist chamber until weighed. All of the organs were weighed in glass stoppered weighing bottles on a chemical balance sensitive to 0.1 mg. The larger kidneys and bladders were weighed to the nearest milligram.

Empirical formulae were obtained for the weights of each organ plotted on body weight and also on body length. The average weights of the organ were determined for each 10 gm increase in body weight or 10 mm increase in nose-anus length and these averages were used in determining the formulae and also in finding the percentage deviations of the calculated from the observed values. All of the straight line formulae were fitted by the method of least squares (Snedecor, '38) and the curvilinear formulae were fitted by the method of trial and error. The formulae are valid only between the limits indicated for each.

KIDNEYS

The weights of the two kidneys are shown plotted on body weight in figure 1. The dots represent the weights of the two kidneys of the male fetuses; the circles, the female kidneys. The dots enclosed by a circle represent those of the male newborn puppies, and the double circles, newborn female kidneys. These cases seemed to be best fitted by two straight line curves. These lines were drawn from the following formulae:

$$Y = 0.0062\ X - 0.0163, \text{ from 3 to 39 gm and,}$$
$$Y = 0.012\ X - 0.244, \text{ from 39 to 440 gm.}$$

In the above formulae, Y represents the weight of the two kidneys in grams and X, the body weight also in grams. The

average percentage deviation of the calculated from the observed values for the first formula or from 3 to 39 gm of body weight is 0.71%. The average percentage deviation for the second part is 6.37% or the average percentage deviation for the entire growth is 4.63%.

The weight of the two kidneys as a percentage of the body weight was determined for each specimen and these percentages were plotted as were the kidney weights in grams. For the first 200 gm increase in body weight, these percentage values were so very irregular that no curve could be drawn

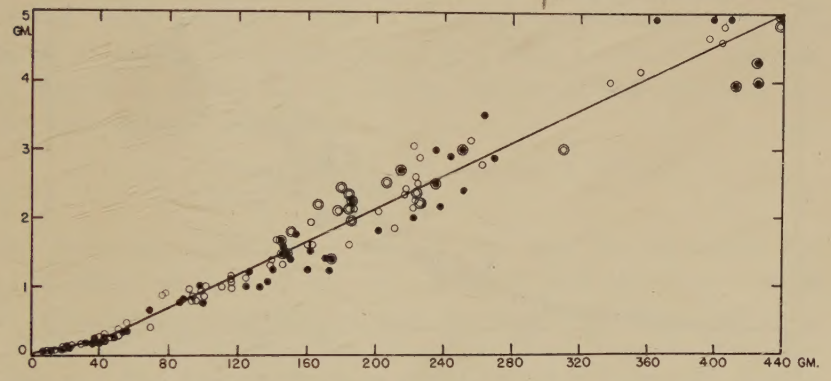

Fig. 1 The weights of the two kidneys are shown plotted on body weight. Both weights are in grams. The dots represent kidney weights of male fetuses, the circles, weights of the female fetal kidneys and the dots enclosed by circles, newborn male and the double circles, newborn female kidneys. The formulae from which these lines were drawn are given in the text.

to represent them with any degree of accuracy. The curve best fitted to these cases would have been an irregular sinusoidal curve rising from about 0.3% to 1.1%. From 200 to 440 gm of body weight these percentages were more regular and they averaged 1.13%.

The weights in grams and also the percentage weights of the newborn kidneys were well fitted by the formulae. Both the absolute weights and the percentage weights of the newborn puppies were scattered among the weights of the fetal dogs. As have been described in the earlier papers,

these dogs were mongrels of all sizes and hence the size of the newborns varied a great deal. The relation of kidney weight to body weight however, seemed to be similar in both the fetal and newborn dogs.

The same kidney weights in grams were plotted on nose-anus length measured in millimeters, and in figure 2, the dots, circles and enclosed dots and double circles have the same connotation as in figure 1. During the first part of this growth of the kidneys, plotted on body length, the kidney weight increased as an exponential function of the

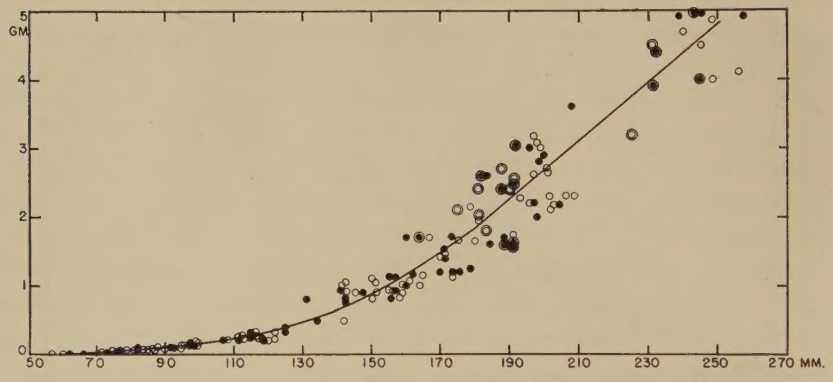

Fig. 2 The weights of the two kidneys in grams are shown plotted on body length in millimeters. As in figure 1, the dots represent males, the circles, females and the enclosed dots and circles, newborn male and female kidneys. The empirical formulae from which these curves were drawn are given in the text.

body length making the first part of this line a curvilinear line. After 180 mm of body length the increase seemed to be a straight line. Two formulae have been derived rather than a more complicated single formula for the entire growth cycle.

The formula from 60 to 180 mm of body length is:

$$Y = \frac{(0.1\ X)^{4.2}}{100600} - 0.0081,$$ and from 180 to 250 mm of body length,

$$Y = 0.044\ X - 6.101.$$

In the above formulae, Y represents the weight of the two kidneys in grams and X, the nose-anus length measured in milli-

meters. The average percentage deviation of the calculated from the observed values for the first formula is 6.30% and for the second, 4.05% or an average percentage deviation for the entire curve of 5.63%. These two curves do not fit the cases quite as well as the formulae for the kidney weights plotted on body weight, but either set of formulae give a fairly accurate estimate of the kidney weights from either body weight or body length.

The percentage weights of the kidneys were more uniformly distributed when plotted on body length than on body weight. The lines are not shown but they were straight lines, the first rising more rapidly. The individual percentages were almost as closely grouped around the lines as are the weights in figure 2. From 55 to 165 mm the empirical formula for the percentage weights is:

$$Y = 0.0074\ X - 0.18, \text{ and from } 165 \text{ to } 260 \text{ mm},$$
$$Y = 0.0012\ X + 0.8438.$$

In these two formulae Y and X are used to represent the percentage weight of the two kidneys and the body length in millimeters respectively. The average percentage deviation of the calculated from the observed values for the first formula is 8.53%, and for the second 3.21%, with an average of 6.40% for both curves.

The growth of the kidneys during the fetal period is very similar in the dog and in the cat. They grow more slowly at first and then early in the fetal period there is an acceleration in the rate of growth. In both animals, the weights of the two kidneys tend to follow the increase in body weight more closely than the increase in body length. In the cat the kidneys increase as a function of the 1.2 power of the body weight. The growth of the dog kidneys against body weight is shown in figure 1 as two straight lines, with the first part of the curve increasing a little less rapidly than the second or later part. The kidney weights increase more slowly at first and then at an increased rate when plotted on body length in both animals.

The percentage weights are likewise very similar in the dog and cat. Both begin at between 0.2 and 0.3% of the body weight and increase rapidly at first and then more slowly to around 1.0% of the body weight at birth. Liesenfield, Dahmen and Junkersdorf ('27) give the percentage weights of the two kidneys for three fetal and one newborn dog. Their percentages are higher for they give a range of from 0.36% in the 40-day-old fetus to 1.58% for the one newborn dog. There are differences in the percentage weights for the adult dog's kidneys. Kunkel ('30) gives the weight of the kidneys for 9 dogs. The percentage weights of these kidneys average 0.73% or 1.55 times the percentage weights given by Junkersdorf ('25) who finds the kidneys averaging but 0.47% of the body weight in the adult dog. Scammon ('33) gives 0.46% for the relative weight of the adult human kidneys. In the adult cat the two kidneys average 0.69% for the females and 0.74% for the males (Latimer, '39). In general, the percentage weights of the kidneys increase during the fetal period in man, in the dog and in the cat, reaching their maximum at birth and then decreasing to the adult level.

After the two kidneys had been exposed to view and before they were disturbed or moved, their relative levels were observed and recorded for 49 specimens. The right kidney was more cephalad in 32 puppies or in 65% of these specimens in which the levels were recorded. The left kidney was higher in but 4 cases or 8%. These differences in level ranged from "slightly higher" to as much as 3 mm in one specimen, which happened to be a newborn dog. The two kidneys were at the same level in 13 specimens or in 27% of the cases.

The kidney levels as recorded for the cats (Latimer, '39) show the right kidney also more cephalad in 74.3% of both fetal and adult cats. In the adult female cats the right kidney was found higher in 88.5% of the cases. The human right kidney is usually slightly lower than the left, "probably due to the presence of the liver" (Goss, '48, p. 1264). In both the cat and the dog the liver lies more on the right side as it does in man, and in these 4-footed animals the right

kidney is usually higher. Can this difference in man be due solely to his upright position?

MESONEPHROS AND RENAL FAT

In the smallest 59 fetuses a pair of mesonephroi was found in all but one specimen. These were small masses just inferior to the inferior pole of the kidneys. They varied in weight from 3.5 to 20.9 mg. Three mesonephroi were found in fetuses weighing above 31 gm, or in fetuses weighing 69.0, 94.0 and 111.0 gm. In a male fetus weighing 129.5 gm a small mass was seen at the inferior pole of the kidneys but it was so small that it was not weighed. In the specimens above 129 gm, no remains of the mesonephros was seen.

No fat, at least in appreciable quantities, was observed around the kidneys in specimens under 26 gm of body weight. At about 26 gm of body weight, fat began to be noticed, first at the hilum and lateral border of the kidneys and then by 150 to 160 gm of body weight both perirenal and pararenal fat was easily recognized. There was a good deal of variation in the amount of fat due very likely to the condition of the mother dogs, hence the above weights are approximations. In all cases however the fat appeared at the hilum and lateral borders first.

URINARY BLADDER

The urinary bladder was removed with the ureters and the urethra severed close to the bladder and all fat removed from the bladders if there was any present. It was weighed after the contained urine had been removed. The weight of the bladder in grams is shown plotted on body weight, also in grams, in figure 3. The two straight lines representing the increase in weight of the bladder were drawn from the following empirical formulae:

$$Y = 0.0009\ X + 0.0091, \text{ from } 10 \text{ to } 70 \text{ gm, and}$$
$$Y = 0.0013\ X - 0.0195, \text{ from } 70 \text{ to } 440 \text{ gm.}$$

In both of these formulae Y represents the weight of the bladder in grams, and X the body weight also in grams. The

dots, circles and enclosed dots and circles represent fetal males and females, and newborn males and females respectively as in the first figure.

The average percentage deviation of the calculated from the observed averages for the first formula is 7.33% and for the second formula, 3.92%, or an average deviation for the two curves of 5.34%.

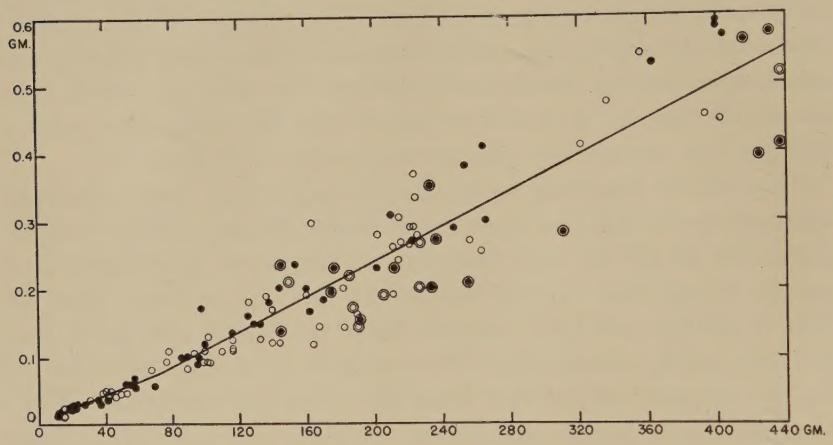

Fig. 3 The weights of the empty bladder plotted, on body weight, with both weights in grams, are shown in this figure. As in the first figure, the dots represent males, the circles, females and the enclosed dots and circles represent newborn males and females respectively. The formulae from which these curves were drawn are given in the text.

The percentage weight of the bladder was determined for each specimen and these percentages were plotted on body weight (not shown). From 3 to 30 gm of body weight the bladder was relatively a little heavier, averaging 0.155% of the body weight. Above 30 gm of body weight, there was some variation of course, but the bladders averaged 0.114% of the body weight throughout the latter part of the fetal period and in the newborn pups. The average percentage weight of all of the bladders is 0.127% of the body weight. All of these weights are for the empty bladder. Each blad-

der was opened and the contained urine removed before weighing.

The same bladder weights were plotted on body length and again this growth can be shown by two straight lines. When plotted on body weight or on body length the rate of increase of the bladder is slightly greater in the latter part of the period. The two straight lines in figure 4 were drawn from the following empirical formulae, determined by the method of least squares:

$$Y = 0.0008\ X - 0.0444, \text{ from } 60 \text{ to } 133 \text{ mm, and}$$
$$Y = 0.003\ X - 0.337, \text{ from } 133 \text{ to } 200 \text{ mm.}$$

In the above formulae Y represents the weight of the bladder in grams and X, the nose-anus length measured in millimeters. The dots, circles and enclosed dots and circles are used as in the preceding figures.

The average percentage deviation of the calculated from the observed weights is 6.16% for the first formula and, 2.80% for the second or an average of 4.48% for the entire growth.

The increase in rate of growth in the latter part of the period is much more marked when the weights are plotted on body length as is seen by comparing this figure with figure 3. The last formula in this figure is valid only to 200 mm. The heaviest bladders as shown in this figure would be above this line were it continued. This group is composed of both fetuses and newborns.

The percentage weights of the bladders were plotted on body length as they were for body weight. Again the first part of the period shows a good deal of irregularity and then above 110 mm of body length they again form a horizontal line, with individual variations of course. The distribution of the individual cases is much like that observed when they are plotted on body weight.

The condition of the bladder was noted on the reverse of each original data card as the parts were removed and weighed. In the smallest specimens, or those under 5 gm of body weight, the bladder was not completely filled but from

5.3 gm on, most of the bladders were full. In many of these smaller specimens the bladder was full and it extended up as far as the umbilicus. In some of the specimens over 6 gm in body weight the bladder was empty. Does this mean that the urine had already been voided into the amniotic fluid? In fetuses with the following body weights the bladder was empty: 6.8, 94, 95, 111, 116 and 160 gm, and in the following the bladder was partly filled: 69 and 160 gm. In the last two, the wall was heavy and looked as though it had been collapsed.

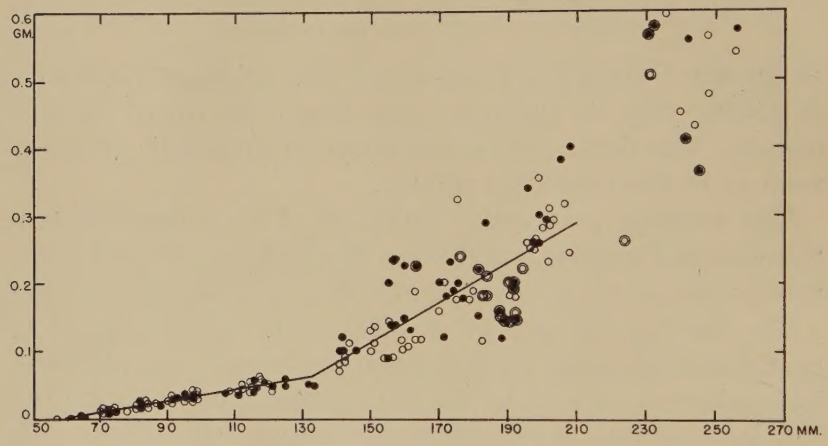

Fig. 4 The weights of the empty bladders, in grams, are shown plotted on body weight in millimeters. The dots represent fetal males, the circles fetal females and the enclosed dots and circles, newborn males and females respectively. The empirical formulae used in drawing these curves are given in the text.

Many of the newborn bladders were large but collapsed. There was but one bladder under 60 gm of body weight which was empty and this was in a specimen of 6.8 gm of body weight. The next one was in a fetus of 69 gm of body weight and it very certainly had been emptied for the wall was heavy and of good size but there was nothing in the bladder. It is easy to think of specimens of 60 gm of body weight and above with bladders which have emptied their contents into the amnionic fluid but the one specimen of but 6.8 gm of body weight seems

rather small to have collected a bladder of urine and to have already emptied it. The bladder was rather small and it may not have as yet stored any urine. The frequency of an empty bladder above 60 gm of body weight seems to indicate that by the time this size is attained, the bladder does empty its contents into the amniotic cavity.

There are ample descriptions of the structure, relations and some estimates of the capacity of the bladder but the only weight of the bladder which is known is given by Peter ('27) quoting from an earlier work of Huschke. This weight of the bladder in the human newborn is 6 gm.

SUMMARY

Empirical formulae are given whereby the weight of the two kidneys or the weight of the empty bladder may be estimated from either body weight or body length.

The percentage weights of the two kidneys are very irregular when plotted on body weight for the first 200 gm and after that they average 1.13% of the body weight. The same percentage weights plotted on body length may be represented by two straight lines, the first rising much more rapidly. Empirical formulae are given for these lines.

The right kidney is more cephalad in 65% of the specimens.

Mesonephroi were found and weighed in the 59 smallest specimens. No renal fat was observed in any specimen under 26 gm of body weight. The fat was observed first at the hilum and lateral border of the kidneys. In specimens above 150–160 gm of body weight both perirenal and pararenal fat was easily recognized.

The percentage weight of the bladder is irregular at first but after 40 gm of body weight it averages 0.114% of the body weight. It becomes filled with urine and then in some of the larger fetuses it evidently had been emptied into the amniotic cavity.

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AGING CHANGES IN THE TUNICA MEDIA OF THE AORTA [1]

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TWENTY-FOUR FIGURES

Certain changes, such as loss of elasticity, fibrosis, calcification, and increase in fat content, that take place in the life history of elastic arteries have been the subject of many studies. Few studies have been made in recent years on the structural pattern and relationships of the several components of the arterial wall. Because of its relative simplicity, the aorta of the mouse is a favorable one for the study of aging. In this investigation the life history of the tunica media has been studied with the emphasis on the aging of the elastic membranes, reticular or collagenous fibers and the ground substance. It has been found that hoops of reticular (collagenous) fibers are closely applied to the elastic membranes of the tunica media and that they become more prominent in old animals. Elastic and collagenous interlamellar fibers increase in number with age. The width of the elastic membranes themselves increases up to 20 days of age and then remains fairly constant. A greater amount of interlamellar elements account for the thickened wall in older animals.

MATERIAL AND METHODS

The data which are presented in this report are based mostly on observations made on the aortae of mice of the C_3H and C57 strains obtained from the Roscoe B. Jackson

[1] This work was done in part under a grant from the United States Public Health Service.

Memorial Laboratory. In all, the aortae of 58 mice were studied: 19, newborn–72 hours; 10, 20–102 days; 14, 141–391 days; 10, 400–495 days; 5, 501–760 days. Aortae of cats, guinea pigs (newborn, 10 days, 2 and 3 months, pregnant adult) and rats were compared with those of the mice.

Tissues fixed in Helly's solution were prepared for general histological observations. Certain tissues fixed in ice cold Rossman's fluid were either treated with the periodic acid-leucofuchsin technique of McManus ('48), or stained in a 0.5% solution of toluidin blue. Material fixed in 4% basic lead acetate was stained in toluidin blue (30 minutes in 0.5% solution) or in thionin (40 drops of saturated aqueous solution in 50 cm^3 of water for one-half hour) for the demonstration of metachromasia. Since the toluidin blue used in all of this work was a 0.5% solution, designated as relatively strong by Lison ('36) and therefore not specific, a control slide of three sections of different ages was tried with the stain diluted 1:10,000. Variations in depth of color of the ground substance paralleled those produced by the stronger stain, only the color reactions were weaker. Control slides, on each of which were mounted sections of aortae and ovary, were treated with hyaluronidase [2] (20 mg in 100 cm^3, Wislocki and Dempsey, '48) to see if the metachromatic reactions were due to the presence of hyaluronic acid. Ground substance was also studied in sections of Rossman fixed material mordanted for 30 minutes in 5% ferric chloride or 5% cuprous sulfate and subsequently stained in Mallory's unoxidized hematoxylin (Lillie, '48).

The most successful method for the dissociation of the elastic lamellae with their sheaths of reticular fibers was the immersion overnight of the fresh tissue in a 1% aqueous solution of sodium bicarbonate. Thin pieces obtained by teasing were mounted directly on clean slides rubbed with albumen. They were impregnated with silver and stained in orcein.

[2] The hyaluronidase was obtained through the courtesy of Doctor A. R. McCullagh of the Schering Corporation.

Sections of tissues fixed in Helly's solution, Rossman's fluid and basic lead acetate were impregnated with silver according to Foot's modification of Bielschowsky's technique which was superior to other methods. These were studied unstained, or stained in hematoxylin and eosin, French's modification of Weigert's resorcin fuchsin, Kornhauser's ('43) orcein or Mallory's connective tissue stain. Unimpregnated aortae were also stained in each of these latter solutions. In order to minimize variations in staining procedures, either 3 or 8 sections of aortae of different ages were mounted on one slide and stained. These were compared with the sections separately mounted.

Measurements of the width of the aortae and of the single elastic membranes were made with a Spencer screw ocular micrometer in sections impregnated with silver with or without a counterstain. Twenty measurements of the width of the wall and of the individual elastic membranes were made at nearly equal intervals around the circumference of the vessel in a single section.

The aortae of mice were sectioned at 5 μ; those of other animals were sectioned at 10 μ.

OBSERVATIONS

The aorta of the mouse is composed of the typical three coats. The tunica media was studied since it is the most prominent, for in the mouse the myo-elastic layers characteristic of the tunica intima of most mammals are not developed. No differences were observed in the aortae of male and female animals. There were characteristically present 5 to 6 membranes (average 5.4; table 1) including the inner elastic membrane. Between these membranes were to be found the ground substance, reticular or collagenous fibers, smooth muscle cells and rarely fibroblasts.

Measurements of the aortae of mice

The measurements of the aortae of 34 mice, ranging in age from 2 hours through 713 days are reported in table 1.

TABLE 1

Average measurements (micra) of the total width of the media and of the individual components of the mouse aorta

AGE	1 TOTAL WIDTH OF MEDIA	2 NO. OF ELASTIC LAMELLAE	3 WIDTH OF ONE MEMBRANE	4 ESTIMATED TOTAL WIDTH OF ALL MEMBRANES (2 × 3)	5 ESTIMATED WIDTH OF INTER-LAMELLAR TISSUE (1 — 4)
hours					
2	24.88	5.9	0.77	4.54	20.34
2	30.46	5.8	1.75	10.15	20.31
24 ±	20.51	4.9	1.03	5.05	15.46
24 ±	28.98	5.1	1.15	5.87	23.11
48 ±	33.14	5.9	0.84	4.96	28.18
48 ±	36.29	5.2	1.07	5.56	30.13
72 ±	28.77	5.9	1.30	7.67	21.10
72 ±	31.34	5.4	0.93	5.02	26.32
days					
20	24.02	5.5	3.32	18.26	5.76
20	30.40	4.8	2.28	10.94	19.46
20	32.36	5.0	2.34	11.70	20.66
20	26.48	4.8	2.15	10.32	16.16
20	26.80	5.9	2.11	12.45	14.35
35	36.24	5.8	2.54	14.73	21.51
35	33.65	5.2	2.62	13.62	20.03
35	40.48	5.4	2.15	11.61	28.87
35	37.02	5.6	2.39	13.38	23.64
83	41.38	4.7	2.09	9.82	31.56
337	66.34	5.2	2.63	13.68	52.66
365 +	34.82	5.4	2.22	11.99	22.83
365 +	42.09	5.6	2.68	15.01	27.08
365 +	39.85	5.0	2.12	10.60	29.25
402	51.59	5.4	2.26	12.20	39.39
455	48.80	5.1	2.88	14.69	33.11
455 +	50.11	6.3	2.85	17.96	32.15
455 +	49.61	5.3	2.43	12.88	36.73
455 +	49.39	5.5	2.17	11.94	37.45
457	53.00	5.5	2.33	12.82	40.18
501	57.33	6.0	2.10	12.60	44.73
598	54.04	4.1	2.53	11.89	41.15
602	69.79	5.5	2.45	13.48	56.31
614	62.94	5.4	2.30	12.42	50.52
669	74.86	5.3	2.97	15.74	59.12
713	58.80	4.8	2.21	10.61	48.19

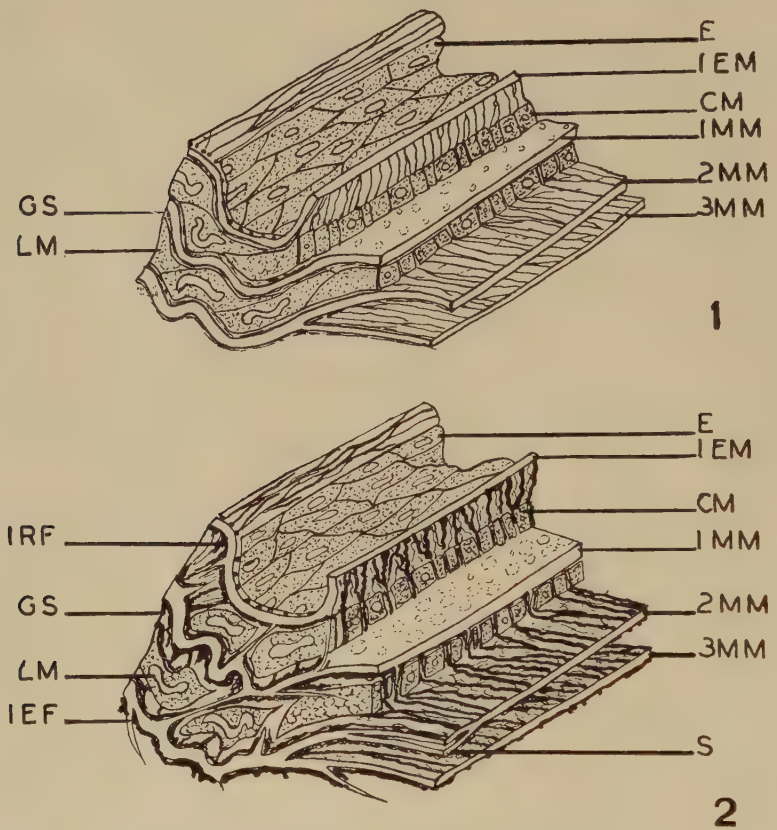
These determinations included the distance from the inner surface of the inner elastic membrane to the outer surface of the outer elastic membrane exclusive of the bordering reticular fibers. As is shown in table 1, this part of the wall of the aorta increased with age: 2 hours–3 days, average 29 μ; 20–83 days, average 33 μ; 337–713 days, average 54 μ. Twenty mice were measured from the tip of the nose to the base of the tail for comparison of the growth in length to the growth in width of the wall of the aorta. Although these data are meager, they indicate that the aortic wall increases in width as the total length of the animal increases (2 hours through 3 days, average 6 animals, 3.3 cm; 20–35 days, 4 animals, 6.3 cm; 365–501 days, 10 animals, 9 cm). After 20 days, this increased width of the tunica media could not be due to an increase in the elastic membranes. The average width of a single lamella was 1.11 μ previous to and including three days; after that time the average width was 2.43 ± 0.0001 μ.

The narrow membranes of the aortae of the young mice were arranged in almost equidistant concentric rings, thrown into narrow, closely set longitudinal pleats upon contraction of the wall (figs. 3, 4, 5). In contrast, the thicker and clear-cut lamellae of the aortae of mice 21 days old and older, were characterized by wide, flat folds (fig. 6). The elastic membranes of the arteries of old mice were in tight waves or broad flat ones in different parts of the circumference of the wall.

Silver impregnation

For these observations material was obtained from newborn, 2, 3, 4, 7, 9, 11, 14, 17, 20, 24, 26 and 28 hour old mice, from mice, 3 through 760 days of age, from rats, 398 and 611 days old, and from 2 cats, 1 young male and 1 pregnant. This material showed that the elastic lamellae of the differentiated aortae were invested with argyrophil fibers arranged circularly (figs. 1, 2).

In the aortae of mice from birth to three hours of age, the membranes were so thin that it was difficult to tell whether the blackened lines (fig. 3) were argyrophilic fibers alone, or argyrophilic fibers bordering on extremely delicate lamel-



Figs. 1 and 2 Diagrams of sections of a young and old aorta to show the changes in aging. Cytoplasm — stippled; reticular fibers — black. C M — cross section of a muscle cell; E — endothelium; G S — region of ground substance, increases in amount in older aorta; I E F — interlamellar elastic fibers; I E M — internal elastic membrane with longitudinal reticular fibers on the inner surface and circular reticular fibers on the outer surface; I R F — interlamellar reticular fibers; L M — longitudinal section of smooth muscle cell; 1 M M — first medial elastic membrane showing honeycomb structure with pale areas far apart in young aorta and closely set in older one; 2 M M — second medial membrane; 3 M M — third medial membrane; S — splitting of second medial membrane in older aorta.

lae. The narrow, clear elastic membrane was seen only after close examination and careful focusing in certain regions. From 4 through 17 hours, the membranes possessed a delicate silver border on one or both surfaces. With the increasing definition of the elastic lamellae in aortae of mice up to three days of age (figs. 3, 4, 5) there was a decrease in silvered material in parts of the wall. At 21 days the hoops of argyrophil fibers were proportionally thin but distinct (figs. 6, 7). A net of fine fibers could barely be seen between them in the teased pieces. As the elastic membranes were unstretched, the reticular fibers on their surfaces were found in characteristically tight folds. At this time there were no silvered fibers extending from these sheaths into the interlamellar spaces. With increasing age the circularly arranged fibers became heavier, the net of fine fibers between them on the surface of the lamella were closer and denser (fig. 8), and fibers appeared between the membranes over the surface of the muscle cells.

Although with aging, the general trend is one of increased density of the argyrophil fibers, in almost all of the aortae there were variations in amount in different regions of one section. Sometimes the width of the blackened network nearly equalled that of the lamella (fig. 9). Similarly, in teased preparations large bundles of silvered fibers appeared to be fraying into finer ones (fig. 8). The interlamellar fibers over the muscle cells were seen as fine strands or grouped as heavier bundles (fig. 9). When silvered sections were subsequently stained in orcein, some of the fine fibers which were partially impregnated with silver were also colored by the stain.

After three days the two surfaces of the inner elastic membrane were quite different in appearance. On the inner surface, the longitudinal reticular fibers appeared in cross section, as a row of dots while the outer surface was covered with circular fibers often extremely thick and similar to those on the medial lamellae (fig. 9).

Sections and teased fragments of the aortae from the rats, guinea pigs and cats were like those from the older mice (figs.

10, 11, 12, 13). In the photograph of a tangential section of the aorta of the 611 day old rat (fig. 12), the lower part of the field shows the network of fibers on the surface of the membrane, and the middle portion, the network around the muscle cells. Figure 13 is a nearly longitudinal piece of the same section. This shows the thickness of the interlamellar fibers between the muscle cells and the interrupted appearance on the membranes.

Periodic acid-leucofuchsin

According to Gersh and Catchpole ('49), the periodic acid-leucofuchsin method for demonstrating polysaccharides combined with protein in ground substance is to be preferred to the use of metachromatic stains. In this investigation both techniques have been used, the former was also employed to stain the reticular fibers.

In these sections the elastic membranes were a greyish ivory or ivory in color and not stained by the fuchsin. In newborn and young animals, the extremely narrow and uncolored elastic membranes could be distinguished from the colored reticular sheaths only with high magnification, good lighting and clear differentiation. In the older animals, the wider membranes could be seen unstained even under low magnification. Elastic membranes in material fixed in Helly's solution were also unstained. In Rossman fixed material, elastic membranes recolored the Schiff reagent only if oxidation with periodic acid was omitted. For controls, sections of ligamentum nuchae of the calf and elastic artery of the cat, fixed in Zenker's fluid, were also studied. The color of the elastic fibers was a pale pink like that of collagen, and much less intense than that of the reticulum. Leblond ('50) finds that the membranes stain with the same intensity with and without previous treatment in periodic acid before the Schiff reagent. According to Lillie ('48) elastin may or may not be stained in the leucofuchsin techniques.

The reticular fibers on the surface of the lamella stained a deep color; with an increase in the amount of ground sub-

stance and in the number of reticular fibers, the aortic wall became more deeply stained. In young aortae, the elastic membranes were bordered by a thin, smooth line (fig. 14). At 70 days short projections extended into the interlamellar spaces. By 102 days and from then on the intensely rose colored reticular fibers could be seen as a lamellar sheath and as interlamellar fibers stretching between the membranes and around the muscle cells (fig. 15). In the oldest mice the thickness of the mat of reticular fibers and the ground substance increased from about one-half to the whole width of the membrane.

In the aortae of newborn mice, dark red granules were seen in the muscle cells; they were absent in all later stages. Since they were digested by saliva, they were considered to be glycogen. Similar granules were found in the aortae of newborn and 10 day old guinea pigs but none were seen in the cells of older vessels.

Staining reactions of ground substance

In this study aging changes were further investigated in the ground substance of the aorta by staining in toluidin blue or thionin and also by mordanting in ferric chloride and cuprous sulfate, and subsequently treating these sections with Mallory's unoxidized hematoxylin. Up to 21 days there was a very fine line of metachromatic color bordering the lamellae (fig. 16). The metachromasia was more intense along the lamellae and outlining the elastic interlamellar fibers in the older aortae (fig. 17). In both thionin and toluidin blue, the aorta of the 760 day old mouse was most intensely colored. Sections of aortae colored with these stains showed a metachromatic reaction even after treatment with hyaluronidase, whereas the follicular fluid in sections of ovary mounted on the same slides lost its color after treatment with the enzyme. The regions which exhibited metachromasia coincided with the places where blackened fibers appeared in the silver technique; variations in one paralleled changes in the other. In 1934, Bensley noted that the metachromatic staining of ground

substance was deeper around the reticular fibers. A slight reaction could be detected in the aorta of the newborn guinea pig. It increased in strength in the older animals and in them, the metachromatic coloring was most evident toward the lumen (as noted by Schultz, '22, in human aortae). Although aging elastic lamellae are said to stain more deeply with basic dyes, in this series the lamellae did not stain at any age.

According to a suggestion made in Bensley's ('34) paper on ground substance, it was thought that the presence of chondroitin sulfuric acid could be demonstrated by its reaction with ferric and copper ions followed by staining in unoxidized hematoxylin. The pale greyish elastic membranes and interlamellar elastic fibers could be seen outlined in blue black or copper blue. In figure 18 the darkest regions are on the surfaces of the muscle cells or between them and the lamellae. In contrast with the slight reaction in the younger aortae, the interlamellar regions of the older ones were deeply colored (fig. 19). The aorta of the 760 day old mouse was stained even more intensely, and in these older aortae there was clear distinction between the unstained or less well stained cytoplasm and the darker ground substance. In these sections the tracheae were deeply colored.

The progressive development with aging of interlamellar fibers and ground substance was further demonstrated by the ease with which single lamellae could be dissociated in young aortae and the difficulty experienced in teasing fragments from older ones. This difficulty was thought to be due to increased numbers of interlamellar fibers and larger amounts of ground substance.

Elastic stains

Orcein and French's modification of Weigert's resorcin fuchsin were used to stain the elastic tissues selectively. Most of the sections impregnated with silver, and all the teased fragments, were counterstained in orcein because its mahogany color offered sharp contrast to the fibers blackened by

silver. A few sections were stained in Weigert's iron-hematoxylin van Gieson method (Huber, '16).

The decrease in depth of color with progressing age was much more clearly seen after staining with orcein than with resorcin fuchsin. The color was deepest at 28 hours and 21 days, slightly less at 70 days, and from 100 through 760 days the membrane became noticeably pale. In contrast to this clear reduction in intensity of color with age in the orcein stained lamellae, a decrease in the intensity of the blue-black color in the fibers stained in resorcin fuchsin was not apparent until the animals were at least one year old. In those sections which were not silvered the bordering reticular fibers were just as deeply colored in orcein as the membranes in the younger aortae.

The appearance of the membranes in the youngest aortae stained in orcein or resorcin fuchsin was strikingly similar to the picture after silver impregnation except that the lamellae were narrower (figs. 3, 20). The membranes of the oldest aortae were distinguished by a less definite appearance. The homogeneous, clear lamellae (fig. 6) characteristic of true vertical sections were interspersed with oblique or tangential sections which in contrast, were more hazy (figs. 22, 23). It was thought that these might be due to an increased number of transverse folds. In his study on the internal elastic membrane of distributing arteries, Strong ('38) suggested that the longitudinal and transverse folds were due to intrinsic elastic qualities of the lamina. Sometimes portions of these oldest lamellae appeared thinner and dissociated into circular fibers (fig. 9). These frequently occurred in regions of anastomosis.

Interlamellar fibers were not seen fraying from the membranes until 70 days of age. After that age there was a marked increase in their length and a varying increase in amount. Some of these interlamellar fibers were much paler than others but much sharper in orcein than in resorcin fuchsin. These ravelled from the membranes singly or in bundles and some enveloped the muscle cells. They were

often shaped like very thin triangles with the bases on the lamellae.

Tangential sections and teased fragments were studied for the structure of the elastic lamella. In thin tangential sections of membranes stained in orcein, resorcin fuchsin, and Mallory's connective tissue stain, there were tiny, pale, roundish areas (fenestrae?) bordered by a more deeply staining substance (figs. 1, 2, 5, 20, 21, 22, 23, 24). In the oldest membranes, these pale areas appeared closer to one another than in the younger aortae, although no measurements were made. This appearance can be likened to a honeycomb or to a thin section of latex sponge where the cavities are filled with a less dense substance which stains either feebly or not at all. It might be expected that these areas would contain ground substance continuous with that between the lamellae. To test this assumption, tangential sections of membranes were studied which were stained by the periodic acid-leucofuchsin method, toluidin blue and the unoxidized hematoxylin following the mordantage in iron and copper salts. The pale areas were observable but much more difficult to see because there was hardly any contrast between them and the denser portions. In cross sections, young elastic membranes were characterized by a smooth homogeneous appearance. This was also true of most portions of the teased fragments from younger aortae. Since this honeycomb structure was seen only in very thin portions in sections and in teased pieces, it was concluded that the membranes are composed of thick or thin mats of networks of elastic fibers, the spaces in the mesh perhaps containing less dense elastic material. A similar picture can be observed in the silvered networks in portions of the tangential sections of the membranes or in teased fragments (figs. 6, 8, 10, 12).

In torn strips of lamellae, in tangential sections and in teased fragments, often, deeply staining (in elastic stains) thick lines ran parallel to and at right angles to the argyrophil fibers (figs. 21, 23, 24). The longitudinal thickened threads appeared as nubbins on the surface of the membranes in

cross sections or as a series of dots on the edges of the fragments (fig. 23). Some of these were interpreted as folds (figs. 21, 23). It was thought that with the decrease in the diameter of the vessel accompanying the fall in pressure and the contraction of the muscle cells, the elastic lamellae were thrown first into the longitudinal ridges which are so characteristic and which appear as narrow or wide undulations depending on age; and secondly into these very much finer and closely set folds which run at right angles to the circular argyrophil fibers. This conclusion was strengthened by the appearance of similar folds in the inner cells of stratified squamous epithelium of a collapsed oesophagus.

In certain regions of teased fragments, particularly of older aortae, straight thickened lines could be seen in the elastic membranes running at right angles to each other (fig. 23). It is possible that the dissociating fluid and the mechanical teasing made more definite the constituent fibers of the lamellae.

Muscle cells

Muscle cells of the younger aortae filled the interlamellar spaces and seemed smoother in outline than those of the older ones. In the 70 day old aortae narrow spaces could be seen between them and the elastic membranes. In the older vessels the cells tended to become more vacuolated and the spaces between them and the lamellae wider. These spaces were bridged by fine fibers from the surface of the muscle cell as if gently pulling the cell toward the membrane. More fibroblasts were present among the muscle cells in the rat and cat aortae than there were in the mouse.

DISCUSSION

Increased fibrosis of the aortic wall, whether in by interlamellar reticular, collagenous, or elastic fibers has been discussed, among others, by Troitzkaja-Andreewa ('31) and Hass ('43). The intimate association of the elastic and collagenous fibers in the aorta has been described by Bloom ('29), Gage

('30), Gross ('50). In some studies on the nature of the aortic wall, the impregnation of fine elastic fibers by silver has been noted (de Kervily, '24; Bloom, '29; Barroso Moguiel, '45). This has been confirmed in the present study. Interlamellar fibers in sections stained in orcein were partially impregnated with silver; in sections stained in orcein alone, the fibrous mat covering the surfaces of the lamellae, so striking in silvered preparations, was deeply colored. It would seem, then, that these techniques do not differentiate the very fine elastic or collagenous fibers.

That the hoops of silvered fibers on the mature elastic membranes are collagenous rather than elastic can be concluded from two observations. In the first place, the elastic fibers fraying from the ivory colored elastic lamellae are also unstained in the periodic acid-leucofuchsin technique in contrast to the rose colored reticulum and ground substance surrounding them and the lamellae. Secondly, the argyrophil fibers are tightly folded on the unstretched elastic membrane in teased fragments and tangential sections. Thus evidence is presented as to their flexibility but non-elasticity. Bloom ('29) likens reticular fibers to coiled springs which resist pull in a tissue and tend to bring it back to its original form. It appears that these circular reticular or collagenous fibers are not only eminently fitted to give added strength to the wall of the elastic artery but also to guard against overdistension.

It is well known that the development of fibers is usually parallel to stress put upon them. Kingsbury ('19) made a clear analysis of the positive relationships between the skeletal needs acting on the connective tissues and their structure many years ago. The categories which he outlined are the following: need of strength — density of tissue; need of elasticity — amount of elastic tissue; direction of stress and strain — arrangements of the fibers. A 4th might be added to these original three: need of resiliency or rigidity — character of ground substance. These principles can be applied to the framework of the aging aorta.

In elastic arteries, blood is sent into vessels whose walls are under continuous longitudinal tension. According to Ophüls ('33), there is a gradual diffuse distension of the artery with aging not only circularly but also longitudinally, due to a progressive deterioration of elastic tissue. These changes which take place are discussed as ones typical of aging colloids by Wells ('33). Elastic arteries, therefore, go through a history in which the elastic laminae are developed, function optimally, and gradually become less effective.

The elastic membranes in the embryonic aorta are preceded by a stage where the ground substance is blackened with silver before they are stained with orcein or resorcin fuchsin (de Kervily, '24; Rainville, '46). Although the elastic lamellae in the newborn aorta are differentiating and stain with elastic stains, there is still in proportion to the width of the membrane, a heavy deposition of silver (fig. 3) which is gradually diminished by 20 days (fig. 6), the time when, in the mouse, the membranes have attained their characteristic appearance and breadth (table 1). Hass ('43) states that the extensibility is greatest in these young networks but that after the removal of mechanical and chemical forces, all elastic systems irrespective of age exhibit identical and almost perfect "retraction." If this is true the different appearance of the folds of the elastic laminae during the life history of the aorta must be largely due to interlamellar factors.

In the older aortae the membranes are less smooth in outline because smaller elastic fibers fray from them into the interlamellar spaces. According to Lansing, Alex and Rosenthal ('50), the percentage of elastin in the human aorta is not significantly changed after the third decade, for up to 20 years of age the percentage is approximately 48% and after that the range is 41.1–44.4%. These investigators did find a marked increase in aspartic acid which may account for the reported increase in basophilia of certain elastic membranes in aging (Wells, '33). This increase has not been de-

tected in sections from the mouse aortae fixed in basic lead acetate or Rossman's fluid and stained in thionin or toluidin blue. In this material the membranes were practically unstained at all ages. The most sensitive indicator of changes due to aging observed in the aorta has been orcein: this dye colors the embryonic fibers in the bird aortae less deeply than resorcin fuchsin (Rainville, '46); in the mouse as well as the bird, after optimum staining of the fibers, there is a distinct loss of stainability with age. While variations were present after resorcin fuchsin they were not as clear cut.

The structure of the elastic lamella is well fitted for expansion. In all stains the basic pattern was that of a honeycomb in which the seemingly more dense portions stained more deeply than the paler regions they surrounded. This structure is seen clearly in the parts of the mouse aorta in which there are tangential sections of the membranes, 2 hours, (fig. 20); 28 hours, (fig. 21); 72 hours, (fig. 5); 365 days, (fig. 22); 405 days, (fig. 23). It is thought that this honeycomb structure is adapted to the stresses acting on the membranes. Even though the magnification is greater in the photographs of the two older aortae, there is a decrease in the darkly staining portions. Further diminution might result in the discontinuous lamellae found in very old aortae. This structure of the elastic lamella is well shown by Hughes ('43) in the chick aorta in the three dimensional diagrams of his type 11 and type 111 arteries. The former is similar to the mammalian aorta with a media composed of concentric elastic lamellae, and the latter possesses them only in the outer half. In both diagrams, the direction of the meshes of the inner elastic lamella is longitudinal. On the other hand, the fibers and the spaces in the network in the medial lamellae of the type 11 artery are transverse and those of type 111 are not oriented. No definite direction could be seen in the arrangement of the pale areas in the mouse aorta. In the dissociated membranes of the pregnant cat aorta where the fibers were more isolated, there was no predominance in one direction. As these fibers are clearly defined only in the older

aortae, there is an apparent age difference in the reaction to the dissociator and teasing.

None of the present observations indicates that the membranes are made up of separate elastic sheets as described by Dees ('23) and Huber ('16). In teased membranes where they have split into two laminae it seemed more probable that it was the region of a branching membrane or an anastomosis. The coarse network of longitudinally directed elastic fibers described and illustrated by Dees ('23) were not found in our preparations.

The muscle cell is the prominent cell in the mouse aorta. Just as in the wall of the alimentary canal it becomes ensheathed with argyrophil fibers. In the aorta these fibers are parallel to the pull of the muscle cells and to the hoops on the membranes. In cultures of heart muscle and aorta, Bloom ('29) describes elastic, reticular and collagenous fibers developing along the lines of muscle pull. In the aging aortae the muscle cells become vacuolated and the space between them and the membrane widens with the increased amount of ground substance. This increased amount of ground substance, closely associated with the elastic and reticular interlamellar fibers would further aid in fortifying the aging wall. According to Ham ('50), the fibroblasts in the walls of the elastic arteries make more amorphous intercellular substance than those of ordinary connective tissue and he suggests that they have some of the properties of chondrocytes. In the mouse aorta, however, it seems more probable that aging changes in the intercellular material have been associated with the muscle cell and not the fibroblast. In those sections stained for ground substance, the color has often been deep on the surface of the muscle cell.

The structure of the aging mouse aorta has, therefore, met the decreasing efficiency in its elastic membranes (1) by increasing the amount of elastin by fine interlamellar elastic fibrils; (2) by augmenting the strength of the wall by circular reticular or collagenous fibers; (3) by maintaining a certain resiliency with an increased amount of ground substance.

SUMMARY

1. The increase in width of the aorta of the mouse is due to the interlamellar components as the membranes double in width before 20 days of age and remain at approximately the same width thereafter.

2. The aortic wall is strengthened by circular reticular or collagenous fibers closely applied to the medial elastic membranes. The density and number of fibers increase with age.

3. Aging is accompanied by an increase in amount of ground substance, and in number and density of elastic and reticular interlamellar fibers.

4. The basic structure of the elastic membrane is thought to be a honeycomb of elastic fibers forming a closely woven mat; the interstices appear to be filled with a less dense material which increases with age.

5. Orcein is a more sensitive indicator of age in elastic membranes than resorcin fuchsin.

6. A real distinction is made between fine elastic and reticular fibers in the periodic acid-leucofuchsin technique.

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PLATE 1

EXPLANATION OF FIGURES

All are cross sections unless otherwise noted.

3 Section of aorta, 2 hour old mouse. A relatively heavy impregnation in the region of the elastic membranes. Helly's solution, Foot-Bielschowsky. × 620.

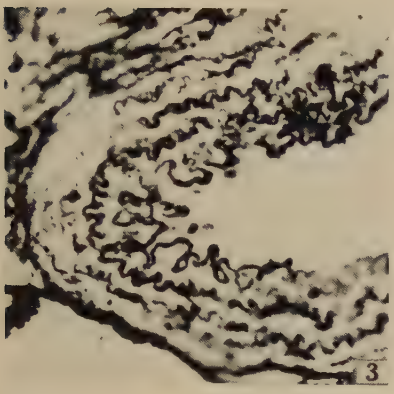
4 Section of aorta, 24 hour old mouse. Diminished amount of silver impregnation. Helly's solution, Foot-Bielschowsky. × 620.

5 Section of aorta, 72 hour old mouse. Very thin silvered lines on elastic membranes. In first and second medial membranes tangential sections show honeycomb structure. Helly's solution, Foot-Bielschowsky — Orcein. × 620.

6 Section of aorta, 21 day old mouse. The differentiated membranes with thin border of argyrophil fibers. Tangential sections of second and third medial membranes with fine networks of blackened fibers. Rossman's fluid, Foot-Bielschowsky — Orcein. × 620.

7 Teased fragment of elastic membrane of aorta, 21 day old mouse. The silvered fibers are tightly crinkled. Fine deposition of silver around pale areas. One per cent sodium bicarbonate, Foot-Bielschowsky — Orcein. × 620.

8 Teased fragment of elastic membrane of aorta, 70 day old mouse. Increase in density of circular fibers of argyrophil network, presence of fine fibrils in between, and around pale areas. One per cent sodium bicarbonate, Foot-Bielschowsky — Orcein. × 620.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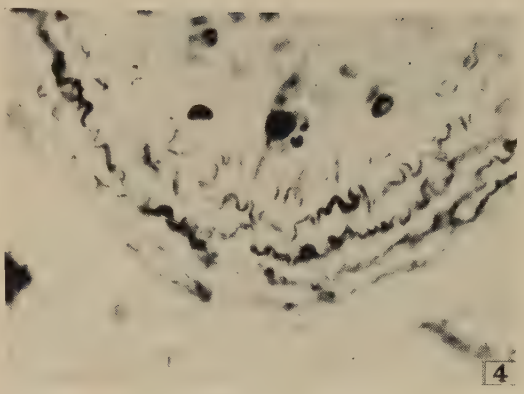
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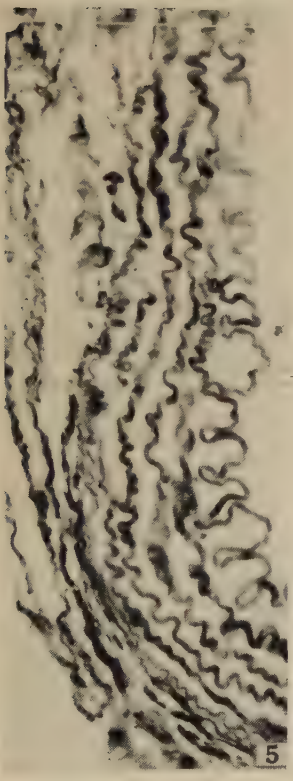
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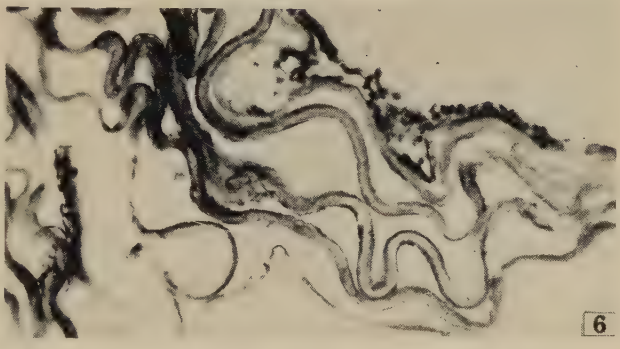
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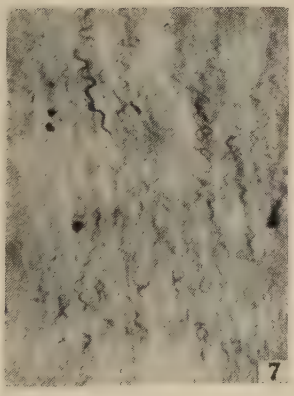
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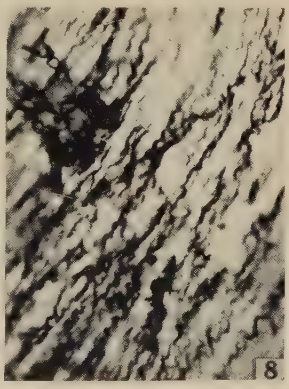
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PLATE 2

EXPLANATION OF FIGURES

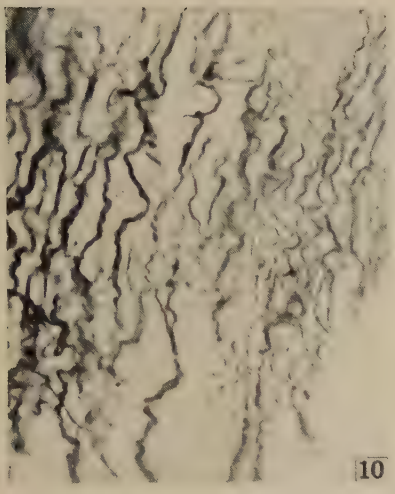
9 Section of aorta, 365 day old mouse. Lumen at left. Cross section of longitudinal fibers of subendothelial layer; thick outer sheath of inner elastic membrane; numerous interlamellar fibers fraying from lamellar sheath over muscle cells; section of second medial lamella, partially tangential of the membrane in region of anastomosis and branching with heavy network of argyrophil fibers. Helly's solution, Foot-Bielschowsky. × 1455.

10 Teased fragment of elastic membrane, aorta of young male cat. Parallel arrangement of circular argryophil fibers on the membrane. One per cent sodium bicarbonate, Foot-Bielschowsky — Orcein. × 620.

11 Teased fragment of elastic membrane, aorta of pregnant cat. Striking increase in density of argyrophil fibers with finer fibrils in between thick ones in focus in some regions. One per cent sodium bicarbonate, Foot-Bielschowsky — Orcein. × 620.

12 Tangential section of aorta, 611 day old rat. In the middle a nucleus of a muscle cell can be seen with the argyrophil fibers running parallel to the direction of the muscle cell. Just below this, a strip of closely set fibers on the surface of the elastic membrane. Zenker's solution, Foot-Bielschowsky — Orcein. × 620.

13 Longitudinal section of aorta, 611 day old rat. Same section as above but a different region. Most argyrophil fibers extend only a short distance from lamellae. Interlamellar fibers stained in orcein barely show. Interrupted appearance of cross sections of circular fibrous sheath of argyrophil fibers. Zenker's solution, Foot-Bielschowsky — Orcein. × 620.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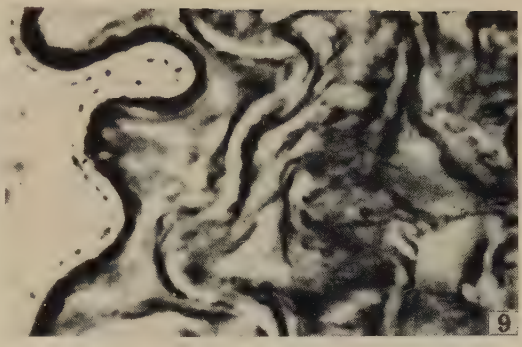
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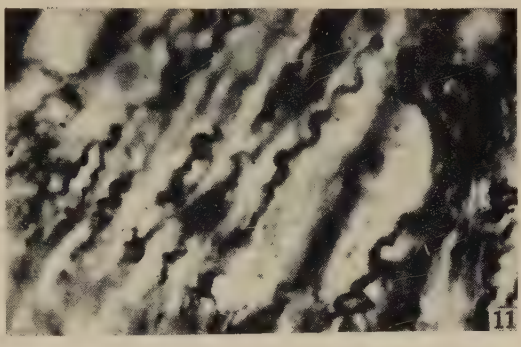
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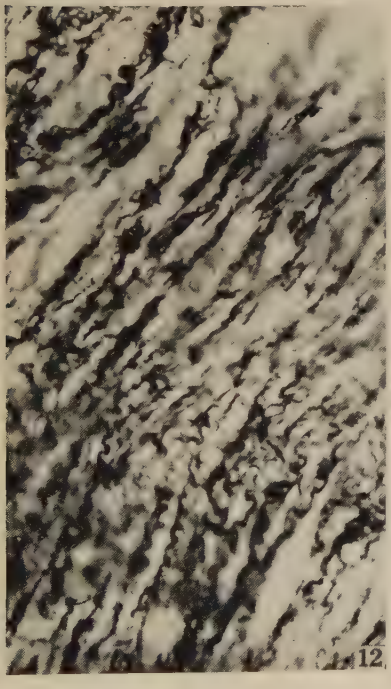
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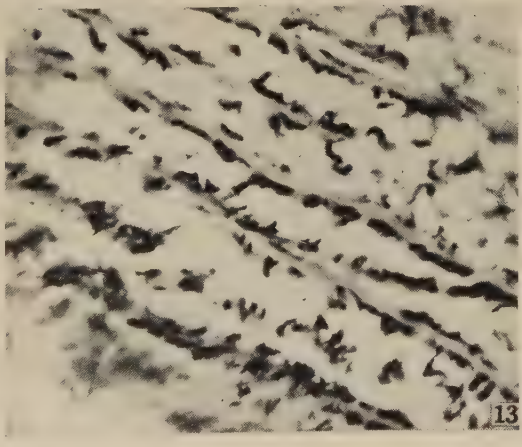
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PLATE 3

EXPLANATION OF FIGURES

14 Section of aorta, 21 day old mouse. Lamellar sheath of reticular fibers, a thin line. The deep ivory color of lamella appears dark in the photograph. Rossman's fluid, Periodic acid-leucofuchsin. × 620.

15 Section of aorta, 429 day old mouse. Lamellar sheath of reticular fibers much thicker. Region between muscle cells and membranes more deeply stained, indication of increase in ground substance and interlamellar fibers. Rossman's fluid, Periodic acid-leucofuchsin. × 620.

16 Section of aorta, 21 day old mouse. Fine line of metachromatic color outlining membranes. Basic lead acetate, Toluidine blue. × 620.

17 Section of aorta, 429 day old mouse. Marked increase in stainable ground substance between muscle cells and lamellae and outlining interlamellar fibers. Basic lead acetate, Toluidine blue. × 620.

18 Section of aorta, 21 day old mouse. Slight amount of ground substance stained in hematoxylin between cells and elastic membranes or on surfaces of cells. Rossman's fluid, Ferric chloride, unoxidized hematoxylin. × 620.

19 Section of aorta, 405 day old mouse. Interlamellar area darkly stained. Deeply stained ground substance surrounds slightly stained cells. Interlamellar fibrils outlined with dark ground substance. Rossman's fluid, Ferric chloride, unoxidized hematoxylin. × 620.

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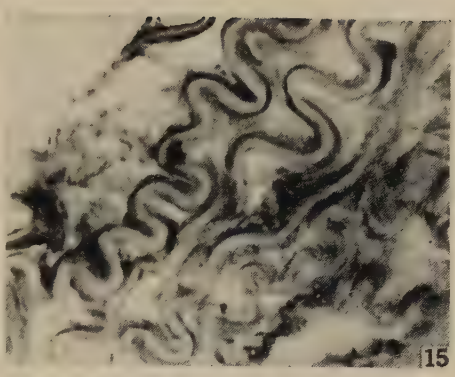
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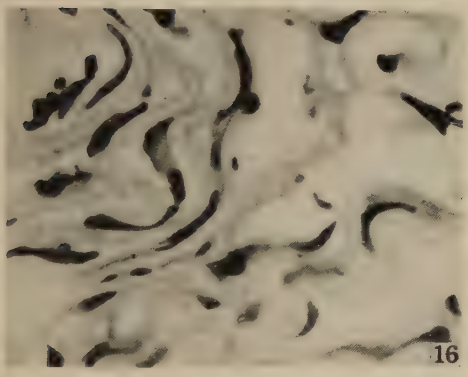
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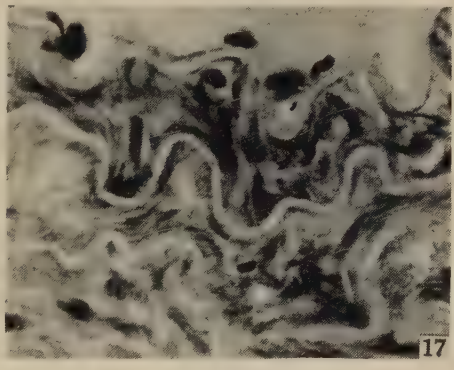
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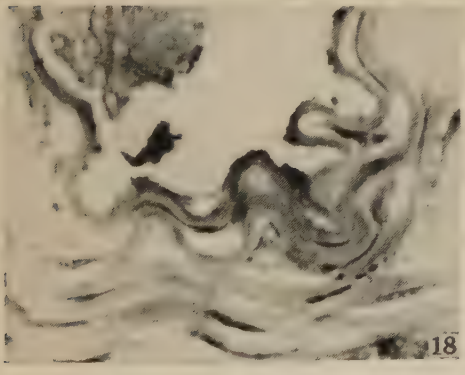
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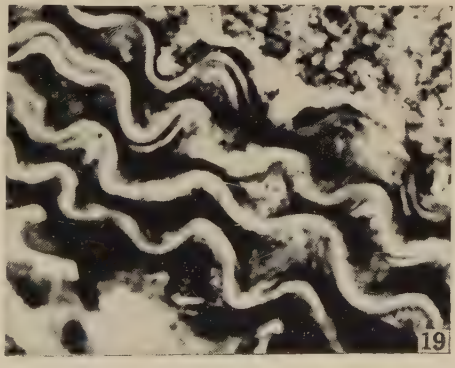
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PLATE 4

EXPLANATION OF FIGURES

20 Section of aorta, 2 hour old mouse. Compare with figure 3. Membranes thinner stained in resorcin fuchsin than in section impregnated with silver. At extreme right, tangential section of membrane with honeycomb appearance. Helly's fluid, Resorcinfuchsin. × 620.

21 Section of aorta, 28 hour old mouse. Inner elastic membrane with honeycomb structure. In medial lamellae fine longitudinal folds. Very fine argyrophil fibers hardly visible. Rossman's fluid, Foot-Bielschowsky — Orcein. × 620.

22 Section of aorta, 365 day old mouse. Compare with figure 9. Fine, pale elastic interlamellar fibers do not show in the photograph. Honeycomb structure of lamellae evident. Helly's fluid, Resorcinfuchsin. × 1455.

23 Section of aorta, 405 day old mouse. Longitudinal folds of inner elastic membrane. Honeycomb structure with pale areas close together at left end of inner elastic membrane and at inner edge of second medial lamella. Rossman's fluid, Resorcinfuchsin. × 950.

24 Teased fragment of aorta, pregnant cat. Thin region of elastic membrane where no argyrophil fibers present. Compare with figure 11. Fibers at right angles to each other stained deeply in orcein. Honeycomb appearance can be seen in several areas. One per cent sodium bicarbonate, Foot-Bielschowsky — Orcein. × 620.

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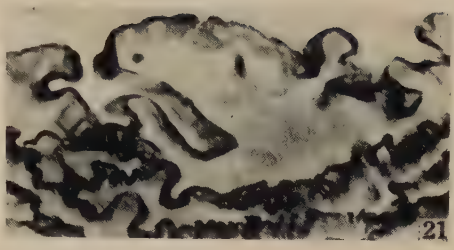
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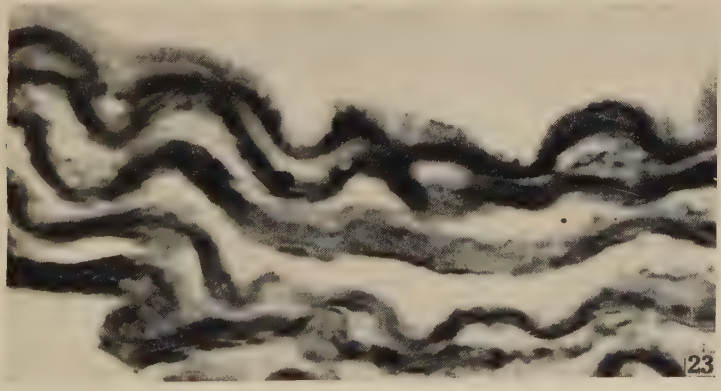
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ENDOCRINE CONTROL OF THE ADRENAL GLAND [1]

JOSEPH D. FELDMAN [2]
New Haven, Conn.

THIRTY-EIGHT FIGURES

INTRODUCTION

The observations to be reported in this paper represent a morphologic investigation of the adrenal gland and its relation to the thyroid and pituitary. The adrenals studied were removed from animals which were used by Hoberman ('50) in an analysis of the endocrine regulation of amino acid and protein metabolism by chemical methods. He employed isotopic glycine which was injected intraperitoneally into rats. From a study of the urinary nitrogen by mass spectrometry the kinetics of amino acid and protein metabolism were followed in animals subjected to a variety of endocrine manipulations. Hoberman's data suggested that (1) an excess or absence of thyroxin affected the adrenal via the pituitary; (2) the action of growth hormone was primarily directed to cellular metabolism and exerted no influence upon the adrenal; (3) adreno-corticotrophic hormone (ACTH) acted directly upon the adrenal.

The present investigation was undertaken to attempt to correlate the morphologic alterations in size and weight and the histochemical changes of the adrenals with the data of Hoberman's study.

[1] This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

[2] Public Health Service Postdoctorate Research Fellow in the Department of Pathology, Yale University School of Medicine, New Haven, Conn. Present address: The Hebrew University — Hadassah Medical School, Jerusalem, Israel.

MATERIAL AND METHODS

Male Sprague-Dawley rats were used, ranging in age from 13 to 15 weeks. Thyro-parathyroidectomy was performed through a midline cervical incision under ether anesthesia. These rats were given drinking water containing 1.8% calcium lactate and 0.2% calcium chloride and sacrificed on the 30th post-operative day. Hypophysectomized animals were obtained from a commercial laboratory [3] and were sacrificed on the 28th post-operative day.

All animals, except 6 fed rats which did not receive isotopic glycine, were handled as follows. They were fasted for 24 hours before the administration of N^{15} glycine and for 48 hours after. One milliliter of a 1% solution of isotopic glycine was given per 100 gm of body weight intraperitoneally. In order to assure adequate urine output, 0.85% sodium chloride solution was injected intraperitoneally at two-hour intervals during the first 10 hours of each day of the three-day fast. One milliliter of this solution was administered per 100 gm of body weight.

For each experiment a control group of rats of similar age was sacrificed at the same time as the experimental group. All animals were killed by etherization and their adrenals were removed immediately, cleaned of fat, weighed on a torsion balance and promptly placed in 10% buffered formaldehyde, pH 7.2. The entire procedure required less than 4 minutes. After fixation for several days, one gland was embedded in paraffin, sectioned and stained with phloxine methylene blue. The other gland was cut on the freezing microtome at 13 to 16 μ; sections were replaced into fresh buffered formaldehyde until used.

The following histochemical procedures were employed:

1. Schiff plasmal reaction. Tissues were immersed in N/10 mercuric chloride for 5 minutes, washed in water, placed in leukofuchsin for 10 to 12 minutes and finally rinsed in three changes of a mixture of potassium metabisulfite and hydrochloric acid (Lison, '36).

[3] Hormone Assay Laboratories, Chicago, Illinois.

2. 2–3 naphthoic acid hydrazide diazo blue B reaction, hereafter designated NAHD. This method was used according to the directions of Ashbel and Seligman ('49). In a previous study it was found to be a stain for both aldehyde and ketone groups, similar to the Schiff plasmal reaction (Feldman, '50).

The above two methods are histochemical tests for carbonyl groups.

3. Sudan stains. Sections were stained in Sudan IV and Sudan black B as recommended by Lison ('36).

4. Examination under the polarizing microscope. Mounted unstained sections of all adrenals were studied through crossed Nicol prisms for the presence and distribution of birefringent material.

5. Acetone solubility. Sections from each adrenal were immersed in absolute acetone for one hour at room temperature and then tested with the above methods.

All sections were washed in running tap water for 15 hours before being used.

Figures for the size of the total gland and cortical zones were obtained as follows. With a micrometer eyepiece measurements of each cortical zone, on both sides of the medulla, were made in the longest and shortest dimensions of preparations stained with Sudan IV and Sudan black B taken from the central portion of each adrenal. The width in micra of the cortical zones of each adrenal was, therefore, determined as the average of 8 measurements. The size in micra of the total gland, including the capsule, and the medulla was determined by averaging the 4 measurements of the longest and shortest dimensions of the same Sudan preparations.

For purposes of measurement, the cortex of non-hypophysectomized rats was divided into three zones according to the criteria described by Bennett for the cat ('40). In Sudan preparations the glomerulosa (presecretory according to Bennett, '40) was readily demarcated from the subjacent zone by the presence of small cells and fine sudanophilic droplets. The middle zone (secretory) was a band of dense sudanophilia

and was regarded as the fasciculata. The innermost zone (post-secretory) was a band of lighter sudanophilia and was regarded as the reticularis.

In hypophysectomized rats there were 4 measurable zones. A narrow glomerulosa was outermost. Subjacent to this was a zone of intense staining reactions and sudanophilia, which, with the third zone, a broad lipid-free region, formed the fasciculata. The innermost zone was the reticularis, irregular in width, deeply sudanophilic and reactive with the various histochemical reagents.

RESULTS

Normal fed rats

Six rats which were fed Purina laboratory chow and plain drinking water and which did not receive glycine or sodium chloride were sacrificed as controls for fasted controls injected with glycine.

Table 1 a presents the average values for the weights and measurements of these glands and the zones within them. The values are not significantly different from those of fasted controls injected with glycine (table 1 a, b, c, d, and e) (figs. 12 and 34).

The histology of the rat adrenal has been described repeatedly and adequately (Crooke and Gilmour, '38; Dalton et al., '43; Zwemer, '36). The following observations are worth noting, however.

(a) The transitional zone, between glomerulosa and fasciculata, was present in 4 of the 6 adrenals in Sudan preparations. It was found in all 6 adrenals when unstained sections were examined in the polarizing microscope (figs. 12 and 34).

(b) With the carbonyl reagents (Schiff plasmal and NAHD reactions) the cytoplasm of the cells of the glomerulosa stained lightly, that of the fasciculata stained deeply, and that of the reticularis stained moderately. With Sudan dyes the cytoplasm remained uncolored.

TABLE 1

		NUMBER OF ANIMALS	TOTAL CORTEX	GLOMERULOSA	FASCICULATA	RETICULARIS	MEDULLA	TOTAL GLAND	PAIRED ADRENALS	ADRENALS MG/100 GM BODY WEIGHT
			μ	μ	μ	μ	μ	μ	mg	mg
(a)	Normal fed rats	6	1041	75	534	431	1512	3640	41	16
(b)	Intact rats given ACTH	6	1101	76	533	492	*864*	3128	*61*	*25*
	Controls	4	1090	72	607	410	*1154*	3374	*38*	*18*
(c)	Thyroidectomized rats	16	*881*	77	488	*315*	1358	*3169*	*37*	*13*
	Controls	12	*1072*	71	563	*437*	1456	*3648*	*45*	*15*
(d)	Thyroxin-treated rats	6	1260	65	710	484	1372	3934	49	22.5
	Controls	3	1127	69	578	479	1338	3637	51	20
(e)	Hypophysectomized rats	6	*565*	*41*	*421*	*103*	1390	*2591*	*16*	*9*
	Controls, unoperated	6	*1103*	*68*	*592*	*443*	1405	*3656*	*52*	*20*
(f)	Hypophysectomized $ACTH_1$	6	*1047*	*76*	*624*	*347*	1588	*3737*	*34*	*21*
	Hypophysectomized $ACTH_2$	6	*758*	*53*	*489*	*215*	1474	*3066*	*52*	*16*
	Hypophysectomized controls	4	*573*	*41*	403	*128*	1371	*2596*	*14*	*8*
(g)	Hypophysectomized thyroxin-treated rats	4	543	42	409	92	1320	2459	12.5	9
	Hypophysectomized controls	3	550	40	408	101	1271	2430	11	7
(h)	Hypophysectomized growth hormone treated	7	529	43	386	153	1353	2599	15	9
	Hypophysectomized controls	7	557	40	415	102	1342	2516	12.5	8

Figures from control and experimental animals within a single group which differ significantly are italicized. P values of .05 or less are considered significant.

(c) Droplets of all cortical cells were Sudan positive but practically all were unreactive with the carbonyl reagents (fig. 20).

Normal fasted controls

The data on weight and measurements of the total gland and cortex of fasted control rats will be presented with each experimental group. Several observations are worthy of note at this point, particularly with reference to the normal fed group.

(a) The transitional zone was a variable feature in Sudan stained sections and in unstained sections examined through crossed Nicol prisms. Within any single group of fasted control animals this zone was present in one or more of the adrenals (figs. 2, 4, 6, 8, 24, 28 and 30).

(b) The staining reactions of cytoplasm and droplets with the carbonyl and Sudan reagents were the same as in the fed group (figs. 19 and 20).

(c) There were usually more sudanophilic droplets throughout the cortex.

(d) Birefringent crystals were also increased, particularly in the fasciculata and occasionally in the reticularis. The doubly refractile crystals in the glomerulosa were of approximately the same number and size in the fasted and fed groups (figs. 24, 26, 28, 30 and 34).

Animals treated with ACTH [4]

Six animals were injected intraperitoneally with 0.2 ml of a 0.5% solution of ACTH in isotonic saline 4 times daily for 7 days. Each rat thus received 4 mg of the hormone. On the 5th day of treatment fasting was started, on the 6th day glycine was injected and the rats were sacrificed 48 hours later. Four control rats received 0.2 ml of isotonic saline 4 times daily for 7 days and were treated in the same way except that they received no ACTH.

Table 1 b presents the data of weights and measurements of these groups. There were no significant differences between

[4] Kindly supplied by Armour and Co., Chicago, Illinois.

the widths of the cortical zones of the groups (figs. 1 and 2). The medulla, however, of the ACTH treated group was significantly smaller than the medulla of the control group. Because of this the total cortex was relatively increased, i.e., it occupied a greater portion of the whole adrenal than did the cortex of the control group. It should be mentioned that the adrenals of the ACTH treated rats weighed significantly more than those of the controls, yet they tended to be smaller in size. The explanation for this is not clear at present.

Histologically the adrenals of the experimental rats revealed several differences from the controls.

(a) The usual three cortical zones were poorly separated from each other due to loss of droplets in the fasciculata and to cytoplasmic staining of all cortical cells by the Sudans (fig. 1).

(b) Sudanophilic droplets were smaller, fewer and less irregular in all zones.

(c) Practically all droplets of the cortical cells were positive with the carbonyl reagents (fig. 21).

(d) A transitional zone was found in none of the adrenals.

(e) Birefringent crystals were diminished and generally coarser than in control sections (figs. 23 and 24).

Thyro-parathyroidectomized animals

Sixteen thyro-parathyroidectomized rats were sacrificed 30 days after operation and after the usual three-day fast and glycine administration. Twelve control animals were autopsied at the same time.

Table 1 c presents the data of the two groups. The adrenal weight and the width of the cortical zones of the experimental rats were significantly less than those of the control animals (figs. 5 and 6). The glomerulosa appeared to be uninvolved in this alteration, and indeed, increased slightly in width compared with controls.

Histologically there were few differences between the two groups except for the obvious decrease in cortical width. In 8 of 16 experimental and 7 of 12 control adrenals a transitional

zone was present. In the glands of the thyro-parathyroidectomized animals the birefringent material tended to be diminished in quantity and coarser in quality (figs. 27 and 28). Otherwise the reactions to the various histochemical tests were the same.

Rats treated with thyroxin [5]

Six rats were injected subcutaneously daily for 7 days with 300 μg of crystalline thyroxin prepared in 0.001 N NaOH. Control rats received subcutaneously 0.001 N NaOH.

Table 1 d presents the data of these groups. Despite the larger size of both the whole gland and the cortex of the thyroxin treated rats, there were no significant differences between this group and the control series in weight or size (P equals 0.1) (figs. 3 and 4).

Histologically the only difference between the adrenals of the two groups was in the amount of sudanophilic and birefringent material. The adrenals of experimental animals contained fewer Sudan positive droplets and birefringent crystals, particularly of the fine type (figs. 25 and 26). In all other preparations the glands could not be distinguished from each other by histological study.

Of interest, also, is the fact that all the adrenals of the thyroxin injected and control animals showed a transitional zone, thus differing from the adrenals of animals administered ACTH.

SUMMARY

When ACTH was administered to fasted rats with intact pituitaries, the adrenals increased in weight without significant increase in size; they lost sudanophilic and birefringent material from the fasciculata and reticularis; their cortical cells contained droplets which were Schiff plasmal and NAHD positive; and the cytoplasm was Sudan positive. When animals were thyroidectomized their adrenals decreased in weight and size significantly and the birefringent material of

[5] Roche-Organon, New Jersey.

the cortex tended to become coarse. When animals were injected with thyroxin their adrenals enlarged in size and increased in weight, but not significantly, and there was loss of sudanophilic and birefringent material from the cortex (table 2).

TABLE 2

	CARBONYL REACTIONS		SUDAN STAINS		BIREFRINGENT CRYSTALS	
	Cytoplasm	Droplets	Cytoplasm	Droplets	Coarse	Fine
Normal fed	++	0	0	++	++	++
Fasted control	++	0	0	+++	++	+++
Intact — ACTH	+++	+	+	+	+++	+
Intact — thyroxin	++	0	0	+	+++	+
Thyroidectomy	++	0	0	+	+	+
Hypophysectomy	+	+	0	+	+++	+
Hypophysectomy ACTH	+++	+	+	+++	+	+++
Hypophysectomy thyroxin	++	+	0	+	+++	+
Hypophysectomy growth hormone	++	+	0	+	+++	+

Composite table of histochemical reactions. The first row represents the baseline of reactions for the remaining rows. An increase in the number of + signs over the number in the first row indicates an increased intensity of carbonyl reactions or an increase of sudanophilic and birefringent material. A decrease of + signs indicates the opposite. The number of + signs is not to be considered a quantitative measurement.

Hypophysectomized animals

Six rats were hypophysectomized at 70 days of age and sacrificed 28 days later after the usual three-day fast and glycine injection. Six intact controls (unoperated) were treated in precisely the same manner.

The data of the two groups are listed in table 1 e. There was striking atrophy of the whole gland and of the cortex of hypophysectomized animals (figs. 7, 8, 29 and 30). The glomerulosa and fasciculata decreased significantly in width, but they occupied almost the same relative portion of the cortex as these zones occupied in the control adrenals. The reticularis atrophied even more than the other zones and the portion of

the cortex which it occupied was much less than that occupied by the reticularis of unoperated controls. The medulla was approximately the same size in absolute values compared with controls, but its portion of total gland was increased because of the diminution in width of the cortex.

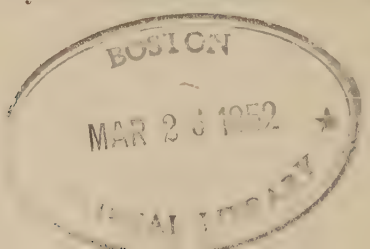
Histologically there was a rearrangement and loss of stainable material in the cortex (figs. 7 and 29). The transitional and reticularis zones were filled with sudanophilic and birefringent material and were deeply stained by the carbonyl reagents. Subjacent to the transitional zone was the remainder and bulk of the fasciculata which was practically devoid of sudanophilic and birefringent substances and which did not react with the carbonyl reagents. The zona glomerulosa was diminished in width and contained relatively few fine Sudan-positive droplets (fig. 17), few or no birefringent crystals and it reacted faintly with the carbonyl stains. It is worth noting that the majority of the droplets in the zones containing them were Schiff plasmal and NAHD positive (figs. 18 and 22).

Hypophysectomized rats treated with ACTH

Nine hypophysectomized rats were given ACTH in the manner described previously. Six of these were sacrificed two weeks following the last dose of ACTH ($ACTH_2$, table 1 f). The remaining three animals were sacrificed 12 hours following the final dose of ACTH ($ACTH_1$, table 1 f). Four rats, hypophysectomized at the same time, and of the same age, served as controls.

Table 1 f presents the data of these groups. On the basis of body weight and on the basis of adrenal size the glands of $ACTH_1$ animals were significantly heavier and larger than those of the $ACTH_2$ group which, in turn, were significantly heavier and larger than those of the control group. It should be remarked that the glomerulosa as well as the other cortical zones of $ACTH_1$ adrenals was as wide as that of unoperated controls (figs. 9, 11, 31, and 33).

Histologically ACTH restored the atrophied adrenals of hypophysectomized rats to a size and microscopic picture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similar to that of the unoperated controls. However, they differed from the adrenals of intact controls in the following:

(a) All zones of the cortex tended to blend together without sharp demarcation, when sections were examined in Schiff plasmal, NAHD and Sudan preparations (figs. 9 and 11).

(b) The glomerulosa was more deeply colored with the carbonyl reagents.

(c) No transitional zone was present.

(d) The fasciculata tended to be wider than was found in non-hypophysectomized control glands and appeared to encroach upon the reticularis, thereby decreasing the latter.

(e) Many droplets in cortical cells were positive with the carbonyl reagents in all zones.

(f) The cytoplasm was colored faintly by the Sudan stains.

(g) Sudanophilic droplets and birefringent crystals were increased beyond the numbers seen in intact controls (figs. 9, 11, 31, and 33).

Hypophysectomized rats treated with thyroxin and growth hormone [6]

Four hypophysectomized rats were injected subcutaneously with 300 μg of crystalline thyroxin daily for 7 days as outlined above. Three control hypophysectomized rats were injected with 0.001 N NaOH daily for the same period and otherwise handled precisely the same as the experimental group.

Six hypophysectomized rats were given 0.2 ml of a 0.5% solution of crystalline growth hormone [6] in isotonic saline intraperitoneally three times daily for three days. Each rat thus received 3 mg of the hormone each day. Fasting was begun on the same day that the hormone was given and isotopic glycine was administered on the second day. Control rats were similarly treated without receiving growth hormone.

The data listed (table 1 g and 1 h) show that the adrenals of thyroxin and growth hormone treated rats were the same in

[6] Kindly supplied by Dr. Alfred Wilhelmi, Department of Physiological Chemistry, Yale Univerity School of Medicine.

weight and size as the glands of control hypophysectomized animals.

Histologically the adrenals of the three groups of animals were identical (figs. 13, 14, 15, 16, 35, 36, 37, 38).

SUMMARY

Ablation of the pituitary caused the adrenal to atrophy in size and weight. The cortex alone was shrunken, all zones were involved, and there was a redistribution of stainable substances. Administration of ACTH restored the atrophied adrenal of hypophysectomized rats to a size, weight and histologic picture similar to that of intact animals. Thyroxin and growth hormone in the hypophysectomized animal had no effect upon the adrenal (table 2).

DISCUSSION

In a previous study it was demonstrated that at the present time there is no test or combination of tests which can visualize cortical steroids (Feldman, '50a). Other investigators have reached similar conclusions (Albert and Leblond, '46; Boscott and Mandl, '49; Claesson and Hillarp, '47; Gomori, '42). It can only be assumed, therefore, that changes in sudanophilia, birefringence and reactions with carbonyl stains noted in these data are related to ketosteroid content, production or release.

The data of the present investigation indicate that the thyroid affects the suprarenal gland through the pituitary. In the intact fasted animal thyroxin caused an increase in adrenal size, an increase in cortical width and a decrease of birefringent material. The measurements of total gland and cortex of the thyroxin treated animals, although larger, were not significantly so compared with their controls. This was probably due to the small number of animals in the control group. When the measurements of thyroxin treated adrenals were compared with the adrenals of all the control animals from all groups they were found to be significantly increased. Similar enlargement of the adrenals in the presence of excess

thyroxin has been reported by others (Deane and Greep, '47; Jaffe, '27; Zwemer, '36).

Thyroidectomy in the intact fasted animal produced the opposite effects on the adrenal. Atrophy of the adrenals in the presence of depressed thyroid function has been reported by Deane and Greep ('47), Leathem ('46), Leblond and Hoff ('44), Seifter et al. ('49), and Zarrow and Money ('49), but was not observed by Williams et al. ('44) or Zeckwer ('38).

The effect of excess or lack of thyroxin was not a direct one, as was revealed by the experiment on hypophysectomized animals. Excess thyroxin in the rat without a pituitary showed no effect upon adrenal size, weight or morphology. In intact rats, however, excess thyroxin and thyroidectomy resulted in enlargement and atrophy of the adrenals. The conclusion to be drawn, then, is that the thyroid acted upon the adrenal through the hypophysis, a pathway of action suggested by Deane and Greep ('47). Scow et al. ('49) and Koneff et al. ('49) presented data which revealed the interrelationship between thyroid and pituitary. Examination of their tables shows that adrenal weight increased or decreased in the presence or absence of thyroxin, provided the pituitary was intact.

This conclusion is consistent with the results on amino acid and protein metabolism reported by Hoberman ('50). His data revealed that thyroidectomy depressed pituitary function which, in turn, depressed the activity of the adrenal cortex. He also found that excess thyroxin increased pituitary function which, in turn, increased the activity of the adrenal cortex (Hoberman, personal communication).

Purified growth hormone caused no gross or microscopic change in the glands of hypophysectomized rats. Growth hormone has been shown to affect cell size and number directly (Simpson et al., '49). In other studies (Feldman, '50b) when growth hormone was injected into hypophysectomized rats, there were marked proliferation, numerous mitoses, and significant weight increase of lymphoid tissue. A similar histological picture was seen in the lymphoid tissues of adrenal-

ectomized rats. These findings indicate that growth hormone does not act through the adrenal.

ACTH was the only endocrine substance which influenced the adrenal directly. In the intact animal, following administration of the hormone, the adrenals increased in weight, though not in total size, and microscopically there was a decrease of sudanophilic droplets, of birefringent crystals and a widening of the cortex. These are alterations indicative of activity (Bergner and Deane, '48; Greep and Deane, '49; Sarason, '43; Weaver and Nelson, '43). In addition cortical cell droplets became positive with both the carbonyl and Sudan reagents.

The most striking changes following the administration of ACTH occurred in the adrenals of hypophysectomized animals. The glands were restored to a size, weight and microscopic picture similar to that of the adrenals of intact control rats. Moreover, there was an increase of sudanophilic and birefringent material beyond the amount seen in the adrenals of intact rats treated with ACTH. In addition the droplets were positive with carbonyl reagents and the cytoplasm was colored by Sudans.

The experiments with ACTH administered to intact and hypophysectomized rats suggest a difference in adrenal response to the pituitary hormone. In the intact animal the adrenals lost sudanophilic and birefringent substances, while in the hypophysectomized rats more of these materials were present. It is possible that in the hypophysectomized animal the rate of discharge of cortical materials is sufficiently reduced to permit accumulation within the cells, or that the demands for their release are diminished.

Cell droplets in adrenals of hypophysectomized rats and of intact and hypophysectomized animals receiving ACTH gave a positive reaction with the carbonyl reagents, whereas the droplets of the other groups of animals did not. The explanation for this alteration in reaction is not clear. It seems most likely that the difference in staining was due to change of the chemical constituents. At most, one can state that the droplets

of hypophysectomized and ACTH treated adrenals contained carbonyl groups (Feldman, '50a).

These observations demonstrate that the entire cortex is under the control of the pituitary. With removal of the pituitary the greatest atrophy occurred in the reticular zone while both the fasciculata and glomerulosa shrank to a lesser extent. Several investigators (Deane and Greep, '46; Deane et al., '48; Nichols, '48; Ducommun and Mach, '49) have reported that the glomerulosa is independent of the hypophysis and that administration of sodium chloride causes an atrophy of this zone. It might be contended that the intraperitoneal injection of salt in the hypophysectomhized rats resulted in glomerular shrinkage. However, the control unoperated animals also received salt and the glomerular zone maintained the same width as in the adrenals of rats which did not.

With administration of ACTH to hypophysectomized rats the zones of the cortex increased to the size of unoperated controls. The data show that the glomerulosa was also restored to a normal width despite administration of sodium chloride. With injection of ACTH to intact rats the glomerulosa became even larger than in adrenals of controls.

In support of these findings Reese and Moon ('37–'38) found the golgi element of glomerulosal cells to be enlarged following injection of ACTH. Jones ('49) also reported variations in the width of the glomerulosa, in mice, in the presence and absence of the hormone.

The transitional zone appears to be a variable and inconstant band. It was occasionally seen in normal and fasted rats, and in thyroidectomized and thyroxin treated animals with intact pituitaries. Yoffey and Baxter ('49) have also commented on the inconstancy of this zone. It was absent in all adrenals of animals receiving ACTH. Following hypophysectomy a narrow zone appeared which was strongly positive for all the histochemical procedures and which was located in the same anatomical position as the transitional zone of intact animals. The function, if any, of this band is unknown.

CONCLUSIONS

The size, weight and morphology of the adrenal is regulated by ACTH. Increased amounts of this hormone cause the adrenal to enlarge and to lose carbonyl-positive, lipid and birefringent material in the intact animal. In the hypophysectomized animal given ACTH the adrenal enlarges but accumulates carbonyl-positive, lipid and birefringent materials. Absence of the hormone, following hypophysectomy, results in atrophy of the adrenal and a loss of these substances.

With the pituitary intact, the adrenal increases in size following the administration of thyroxin; it decreases in size and weight following thyroidectomy. In the absence of the pituitary, the adrenal is not affected by the administration of thyroxin.

Growth hormone has no influence upon the adrenal grossly or microscopically.

The findings of this investigation support the conclusions obtained on the same experimental material by chemical methods.

I am deeply grateful to Dr. Henry Bunting, Department of Pathology, Yale University School of Medicine, for his helpful suggestions and valuable advice in the preparation of this paper.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

Photomicrographs 1 to 16 reproduced at a magnification of 50 × and stained with Sudan black B.

1 Cortex of intact rat injected with ACTH. Absence of transitional zone. Blurring of zones.

2 Cortex of fasted control for 1. Glomerulosa and reticularis demarcated from fasciculata.

3 Cortex of rat injected with thyroxin. Absence of transitional zone in area shown. Width of cortex larger than control (4). Poor demarcation of various zones.

4 Cortex of fasted control for 3.

5 Cortex of thyro-parathyroidectomized rat. Cortex is atrophied compared with control (6). Transitional zone present. Inner portion of glomerulosa contains sudanophilic droplets; outer portion lipid-free.

6 Cortex of fasted control rat for 5.

7 Cortex of hypophysectomized rat. Rearrangement of sudanophilic material into two dense bands. Absence of sudanophilia in glomerulosa and fasciculata.

8 Cortex of intact fasted control rat for 7.

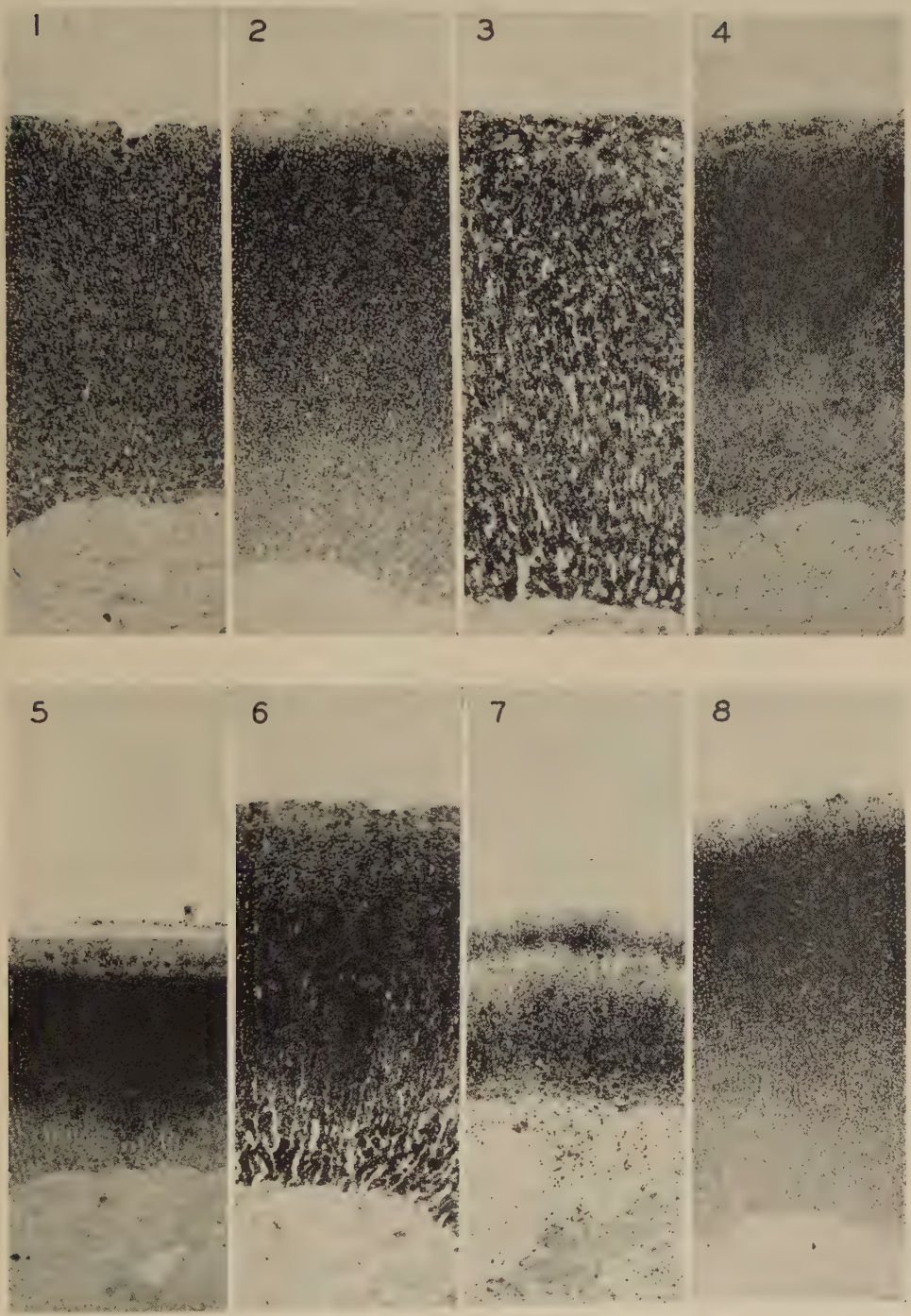
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PLATE 2

EXPLANATION OF FIGURES

Photomicrographs 1 to 16 reproduced at a magnification of 50 × and stained with Sudan black B.

9 Cortex of hypophysectomized rat sacrificed 12 hours after last dose of ACTH. Cortex the same as fasted controls (2, 4, 6 and 8). Absence of transitional zone. Blurring of zones.

10 Cortex of hypophysectomized rat control for 9.

11 Cortex of hypophysectomized rat sacrificed two weeks after last dose of ACTH. Diminution of width of cortex. Transitional zone still absent. Blurring of zones still present.

12 Cortex of normal fed rat.

13 Cortex of hypophysectomized rat injected with thyroxin. Rearrangement of sudanophilic material. Histology the same as hypophysectomized control (14).

14 Cortex of hypophysectomized rat control for 13.

15 Cortex of hypophysectomized rat injected with growth hormone. Histology the same as hypophysectomized control (16).

16 Cortex of hypophysectomized rat control for 15.

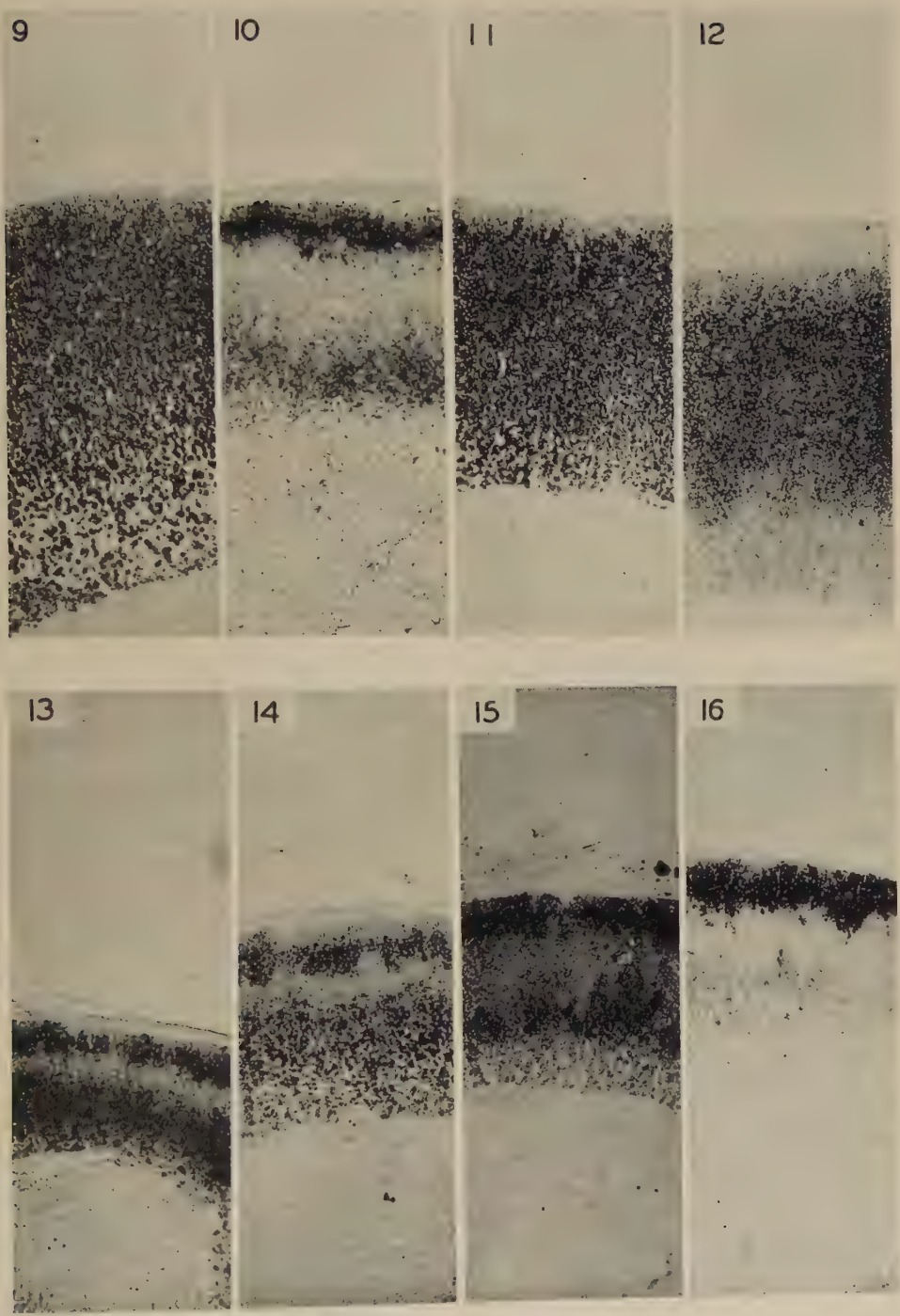
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PLATE 3

EXPLANATION OF FIGURES

17 Glomerulosa, transitional zone and portion of fasciculata of hypophysectomized rat. Glomerulosa practically devoid of sudanophilic droplets. Transitional zone filled with sudanophilic droplets. Stained with Sudan black B. Magnification × 180.

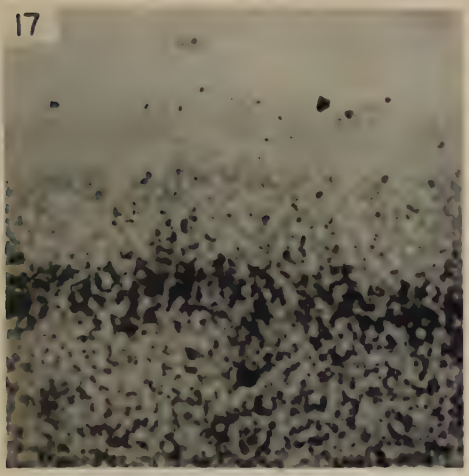
18 Reticularis of hypophysectomized rat. Stained with naphthoic acid hydrazide diazo blue B. Droplets are large, irregular and positive. Magnification × 1140.

19 Fasciculata of fasted control rat. Stained by the Schiff plasmal method. Droplets are uncolored. Magnification × 760.

20 Fasciculata of normal fed rat. Stained with naphthoic acid hydrazide diazo blue B. Droplets are uncolored. Magnification × 1140.

21 Fasciculata of intact rat injected with ACTH. Stained with naphthoic acid hydrazide diazo blue B. Droplets are positive. Magnification × 760.

22 Outer fasciculata of hypophysectomized rat. Stained with naphthoic acid hydrazide diazo blue B. Droplets are positive, and irregular in size and shape. Magnification × 760.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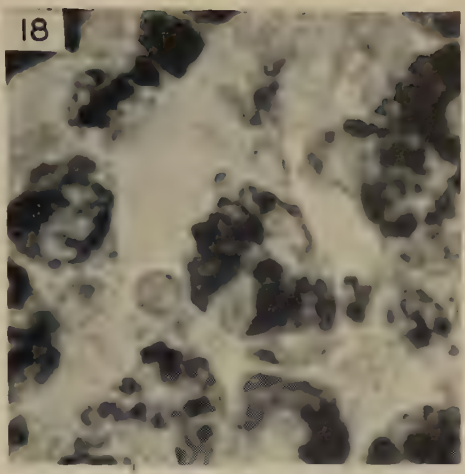
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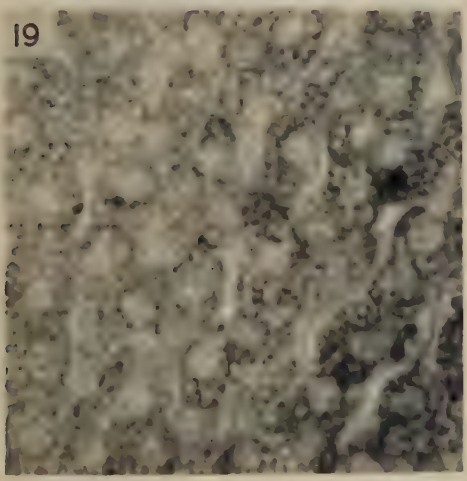
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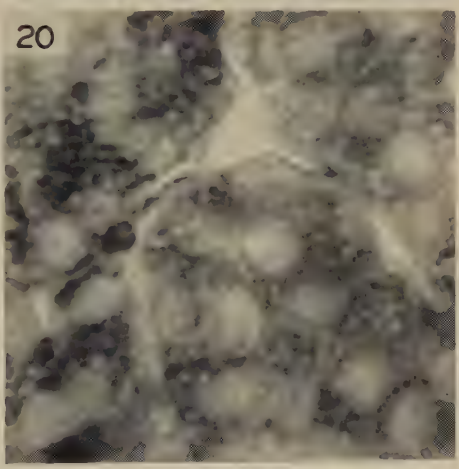
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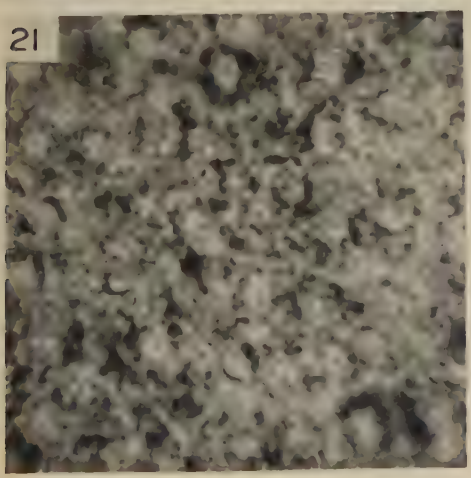
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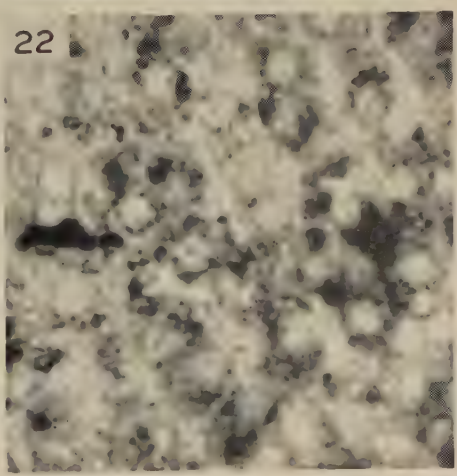
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PLATE 4

EXPLANATION OF FIGURES

Photomicrographs 23 to 38 reproduced at a magnification of 44 × and taken through a polarizing microscope. The width of the cortex, including capsule, is indicated by the white lines at the right of the photographs.

23 Cortex of intact rat injected with ACTH. Birefringent crystals decreased in number and increased in size compared with control (24).

24 Cortex of fasted control for 23.

25 Cortex of rat injected with thyroxin. Birefringent crystals decreased in number and increased in size compared with control (26). Transitional zone present.

26 Cortex fasted rat control for 25. Transitional zone present.

27 Cortex of thyro-parathyroidectomized rat. Diminution in width of cortex, loss of birefringent crystals which are coarse compared with control (28).

28 Cortex of fasted rat control for 27.

29 Cortex of hypophysectomized rat. Distribution of birefringent crystals into two bands. Increase in size of crystals compared with fasted control (30).

30 Cortex of intact fasted rat control for 29.

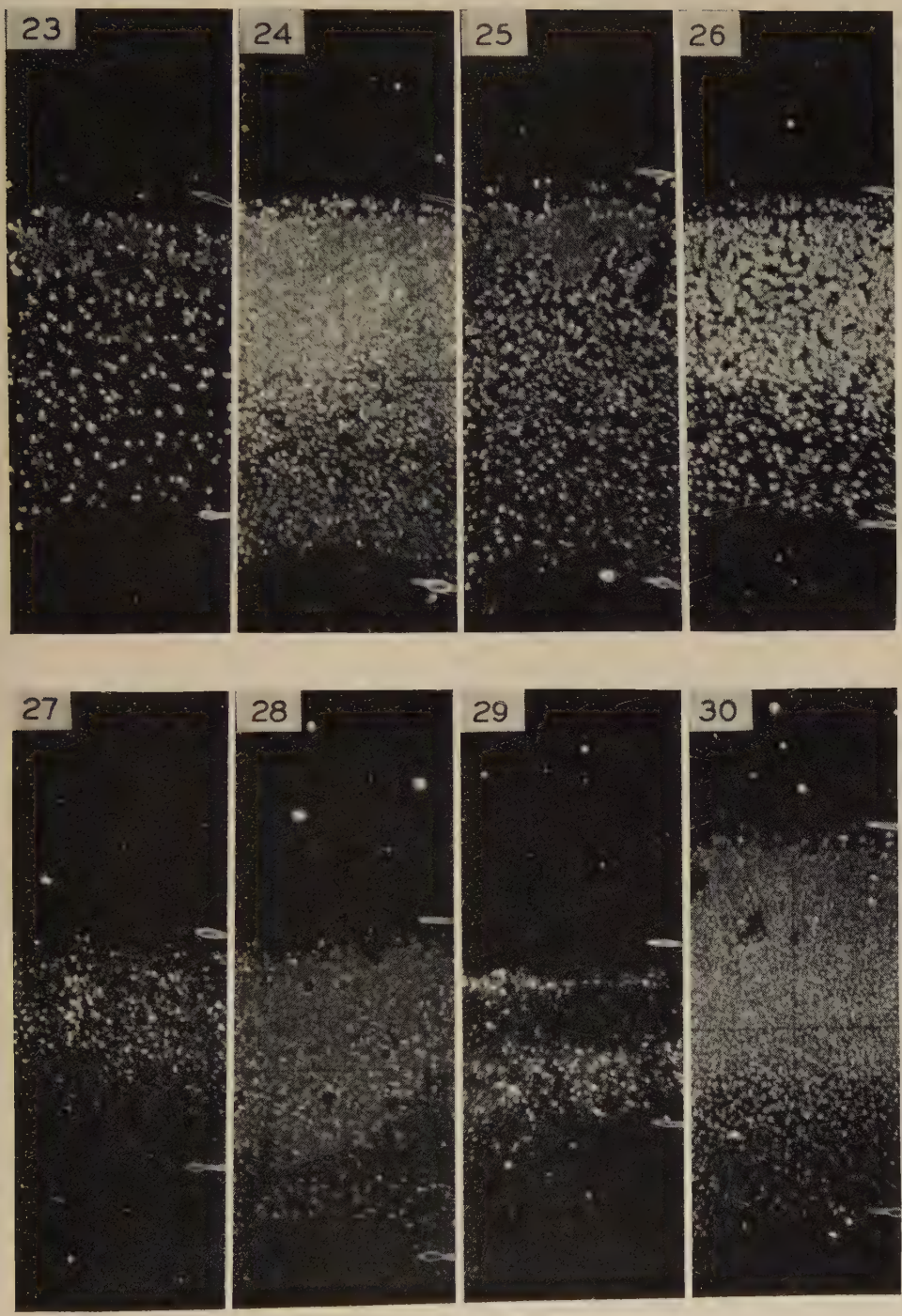
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PLATE 5

EXPLANATION OF FIGURES

Photomicrographs 23 to 38 reproduced at a magnification of 44 × and taken through a polarizing microscope. The width of the cortex, including capsule, is indicated by the white lines at the right of the photographs.

31 Cortex of hypophysectomized rat injected with ACTH to within 12 hours of death. Width of cortex the same as fasted controls (24, 26, 28, 30).

32 Cortex of hypophysectomized rat control for 31 and 33.

33 Cortex of hypophysectomized rat injected with ACTH up to two weeks before death. Diminution of width of cortex compared with 31. Transitional zone absent. Birefringent crystals are numerous and fine.

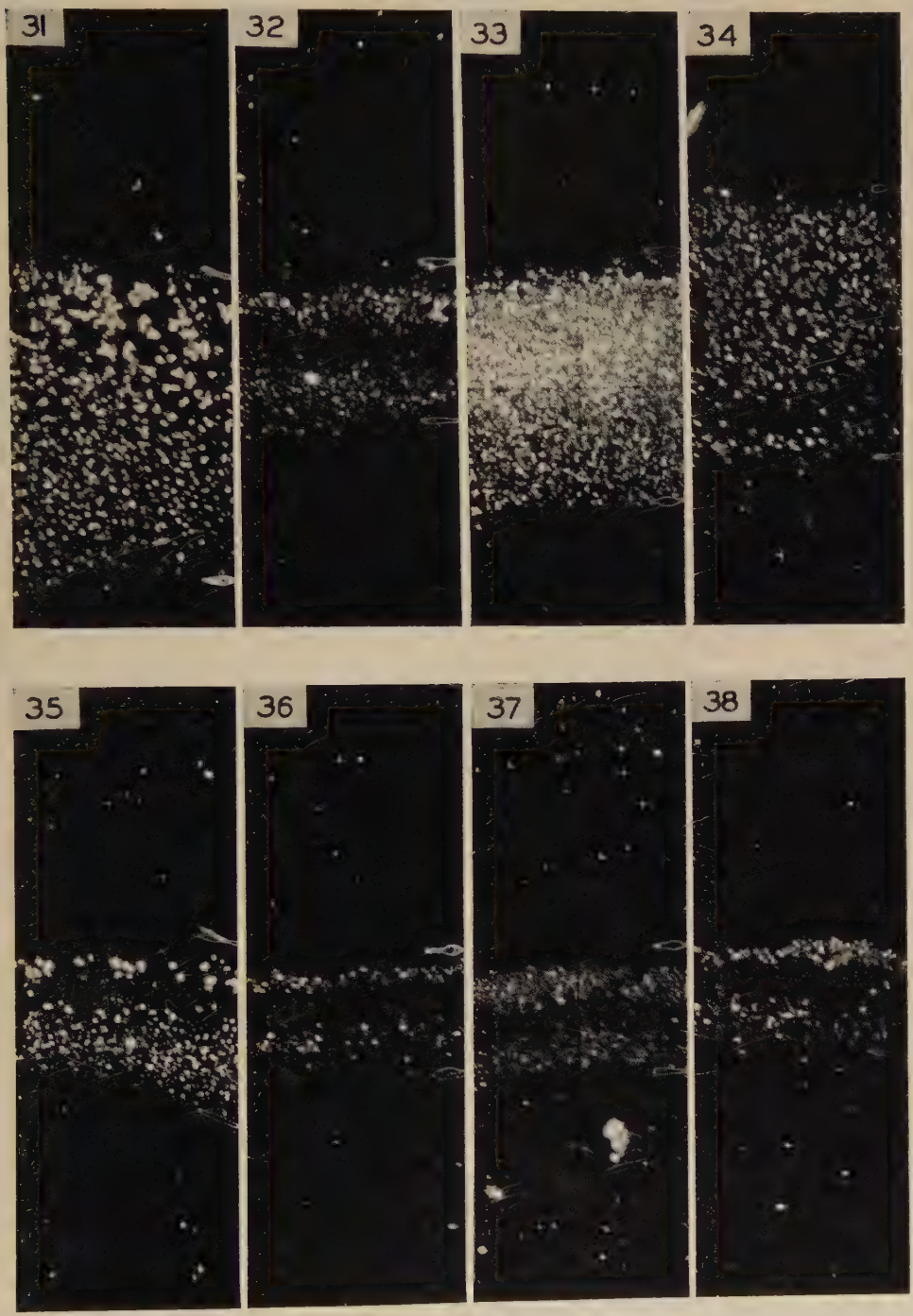
34 Cortex of normal fed rat.

35 Cortex of hypophysectomized rat injected with thyroxin. Distribution of birefringent crystals into two bands. Increase in size of crystals. Same as control (36).

36 Cortex of hypophysectomized rat control for 35.

37 Cortex of hypophysectomized rat injected with growth hormone. Same as control (38).

38 Cortex of hypophysectomized rat control for 37.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LOCALIZATION OF ACID PHOSPHATASE IN FIBROBLASTS[1]

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FIVE FIGURES

INTRODUCTION

Probably no cell in the body has been more difficult to describe satisfactorily than the ubiquitous fibroblast. This is due mainly to the fact that the term fibroblast has been applied to a morphological type of cell which is either a large group of different functional types or a single cell with many functions. Misleading technical results have added to the haze. As an example of this we offer the case of the fibroblast and acid phosphatase. One wonders why the fibroblast is described as only occasionally containing the enzyme. Is this a strain difference or is it a caprice of technique?

To answer this question and to identify the factors which account for these variable reactions, fibroblasts from different locations of the same animal and from different kinds of animals were subjected to controlled acid phosphatase procedures. The results are described at some length because the question of variable reaction to the acid phosphatase technique is certainly not peculiar to the fibroblast.

[1] This work was partially supported by a grant from the Coordinating Council for Cerebral Palsy in New York City, Inc.

SOURCE OF MATERIAL AND EXPERIMENTAL DESIGN

Connective tissue spreads of subcutaneous tissue, muscle fascia and mesentery of adult rats, mice and hamsters were used. In addition hanging drop tissue cultures of fibroblasts from 18-day-old chick embryos and prostate glands of young rats were studied. This material was treated as follows:

1. Fresh unfixed spreads and cultures were placed directly in the glycerophosphate reagent of Wolf, Kabat and Newman, '43 (except for the use pentahydrate sodium glycerophosphate Eastman) at pH 4.7 at 37°C. They were then incubated for varying intervals of time (2 to 72 hours). After washing in distilled water they were immersed in 1:40 dilution of yellow ammonium sulphide for one minute and then examined.

2. Spreads and cultures fixed in ice cold acetone were dipped in distilled water for a few minutes and then incubated in the glycerophosphate reagent for from 2 to 24 hours. They were then washed again in distilled water and immersed in 1:40 dilution of yellow ammonium suphide for one minute.

3. Simultaneous controls were run in all experiments. Some of the spreads and cultures were exposed to an incubating reagent which lacked the substrate sodium glycerophosphate; others were exposed to an incubating reagent which contained the enzyme inhibitor sodium fluoride in a concentration of 0.005 N (Gomori, '41, and Rabinovitch, Junqueira and Fajer, '47). Negative reactions were obtained in all controls. This indicates that our observations were the result of acid phosphatase activity.

OBSERVATIONS

Unfixed fibroblasts (figs. 1, 2, and 3)

The enzyme acid phosphatase was always found to be present in the cytoplasm of unfixed fibroblasts. This was true regardless of either the source of the cells or the length of time that the cells were incubated in the substrate. In contrast to the cytoplasm the presence of the enzyme in the nucleus varied with both the source of the cells and the length of the

incubation time. No enzymatic activity was noted in the nuclei of cultured fibroblasts or in those of the hamster. The nuclei of rat and mice fibroblasts sometimes showed activity depending upon incubation time. Two to 6 hours of incubation seldom produced a result but 24 to 72 hours resulted in a faint to moderate reaction in some of the nuclei.

Fixed fibroblasts

In fixed material it was found that by manipulation one could produce just about any reaction desired. The range as to the location of demonstrable enzymatic activity was from positive in the cytoplasm and negative in the nucleus to negative in the cytoplasm and positive in the nucleus. This complete reversal as to location of enzymatic activity is related to lengths of fixation and incubation time.

In rat and mice connective tissue spreads, fixation for 15 minutes to 3 hours and incubation for 2 to 5 hours showed acid phosphatase activity in the cytoplasm but none in the nucleus; fibroblasts from the same source fixed for 15 minutes to 24 hours and incubated from 18 to 24 hours showed activity in the nucleus but not in the cytoplasm except for occasional cells which showed a few granules.

The tissue cultures and hamster connective tissue spreads fixed for 24 hours showed moderate enzymatic activity in their fibroblast nuclei but none in the cytoplasm.

As expected, fibroblasts treated for intermediate lengths of fixation and incubation times showed results which were variable in that they indicated a reaction which was shifting from positive cytoplasm and negative nucleus to negative cytoplasm and positive nucleus. Both end types with intermediate stages were present.

Mast cells, endothelial cells, smooth muscle cells and perivascular cells

The variable picture obtained in fibroblasts by technical manipulation raised the question as to whether a similar vari-

ation was present in other cell types. To determine this, mast cells, endothelial cells, arteriolar smooth muscle cells and perivascular adventitial cells were subjected to the same procedures. No difference in the localization of acid phosphatase activity was found between the fixed and the unfixed material. The mast cells showed intense enzymatic activity in the cytoplasmic granules but slight or negative activity in the nuclei. The endothelial cells showed intense enzymatic activity in the nuclei but weak in the cytoplasm (figs. 4 and 5). The nuclei of the arteriolar smooth muscle cells had strong to moderate activity whereas positive to negative reactions were observed in the cytoplasm. In the perivascular adventitial cells the activity was intense in the nuclei but only slightly positive or negative in the cytoplasm (figs. 4 and 5). Some unfixed perivascular cells showed some evidence of activity as stained cytoplasmic granules.

DISCUSSION

The above observations remove a bit of the haze surrounding our knowledge of the fibroblast. They indicate clearly that acid phosphatase is always present in fibroblasts. Since this is true only when the material has not been fixed it is obvious that the standard method which utilizes fixed and paraffin-embedded tissues is not only inadequate but is misleading as well. Coupled with the observations of Bartelmez and Bensley ('47), who reported acid phosphatase in the cytoplasm of unfixed and rarely in fixed Schwann cells, it is our conviction that unfixed material should always be used in studies involving this enzyme. This thought should not offend anyone who is more interested in knowing cells than in following standard procedures.

The interpretation as to where the enzyme is located within the cell is a matter for considerable caution. In the technique used, lead phosphate was deposited at the sites of acid phosphatase activity. The sites of deposition were then visualized by transforming the lead phosphate to black lead sulphide. As noted above, in general the enzyme is found in the cytoplasm

in unfixed and in briefly fixed and briefly incubated fibroblasts; in the nucleus in fibroblasts fixed and incubated for a long time.

There can be no doubt that acid phosphatase is always present in fibroblasts nor that it is present in the cytoplasm of the fibroblast. In contrast its presence in the nucleus is suspect. The greater weight of our experimental evidence indicates that the deposit of lead sulphide in the nucleus is due to diffusion, probably of the lead phosphate. An indication of salt diffusion is evidenced in fibroblasts that are fixed for from 15 minutes to 2 hours. After short incubation (2 to 5 hours) these fibroblasts show activity localized as granules in the cytoplasm and absent to faint reactions in the nuclei. After longer incubation (6 to 8 or 14 to 16 hours) many of these fibroblasts show activity evenly distributed throughout the cytoplasm and of moderate intensity in the nuclei. It seems that the longer incubation allows for a shift of lead salt from cytoplasmic granules to the cytoplasm in general and the nucleus. After still longer incubation (16 to 24 hours) most fibroblasts show no activity in the cytoplasm and moderate activity in the nuclei.

We think of this diffusion as occurring through the two surfaces of the cytoplasm, i.e., through both the cell membrane and the nuclear membrane. Material which diffuses through the cell membrane is lost as far as ability to demonstrate it is concerned, that which passes through the nuclear membrane is retained within the nucleus sufficiently long to be demonstrated.

In contrast to the highly questionable presence of acid phosphatase in the nuclei of fibroblasts, there is no doubt that the nuclei of mast cells, endothelial cells, smooth muscle cells and perivascular cells contain the enzyme. No difference in the visualization of acid phosphatase activity was found between the fixed and unfixed cells of this group.

SUMMARY

1. Acid phosphatase activity was noted in the nucleus and the cytoplasm of fibroblasts of the rat, mouse and hamster, and of tissue culture fibroblasts of the rat prostate gland and chick embryo.

2. The localization of acid phosphatase in the fibroblast is dependent upon the degree of fixation and the duration of incubation. In the unfixed fibroblasts the enzymatic activity was localized in cytoplasmic granules and was either absent or slight in the nuclei. After brief fixation (15 minutes to 3 hours) and brief incubation (2 to 6 hours) fibroblasts exhibited the same localization of acid phosphatase as in unfixed fibroblasts. After fixation (15 minutes to 24 hours) and long incubation (18 to 24 hours), fibroblasts exhibited enzymatic activity in the nucleus and either none or traces in the cytoplasm.

3. The observations suggest that acid phosphatase is localized in the cytoplasm of the living cell and that some of the enzymatic activity detected in the nucleus is due to the diffusion of lead phosphate from the cytoplasm.

4. Use of fresh tissues whenever it is technically feasible is indicated to check activity revealed by the standard Gomori acid phosphatase technique.

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PLATE

PLATE 1

EXPLANATION OF FIGURES

All figures are from unfixed tissues incubated in the glycerophosphate-lead nitrate reagent at pH 4.7, washed and then placed in dilute yellow ammonium sulphide. No counterstain.

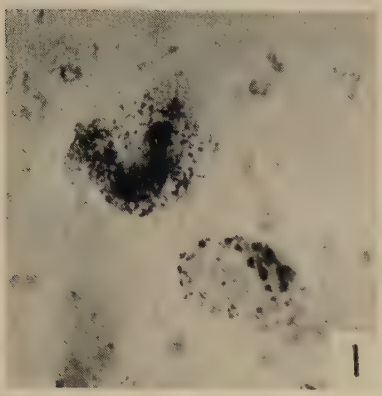
1 Two fibroblasts from the omentum of the rat. Acid phosphatase activity is indicated by cytoplasmic granules. The nucleus of one cell has moderate activity while the other shows no activity. Incubated 24 hours. × 900.

2 Fibroblasts from muscle fascia of the rat. Acid phosphatase activity is indicated by cytoplasmic granules in the perinuclear region and in the cytoplasmic processes. Incubated 4 hours. × 900.

3 Fibroblasts from the omentum of the rat. See figure 2. × 900.

4 Blood vessels from the omentum of the rat. In the endothelial cells in lower part of figure acid phosphatase activity is strong in the nuclei but weak in the cytoplasm. In the perivascular adventitial cells in the upper part of the figure the enzyme activity is intense in the nuclei and moderate in the cytoplasm. The fibroblast between the two large vessels has cytoplasmic granules as noted above. Incubated 4 hours. × 900.

5 Blood vessels from the omentum of the rat. Pointer is on a perivascular adventitial cell nucleus having acid phosphatase. Note strong reaction in endothelial cell nucleus to the left of the pointer. Weak but definite phosphatase activity is present in the endothelial cell cytoplasm. Intense to moderate reactions are apparent in the nuclei of smooth muscle cells of arteriole in upper part of figure. Incubated 4 hours. × 900.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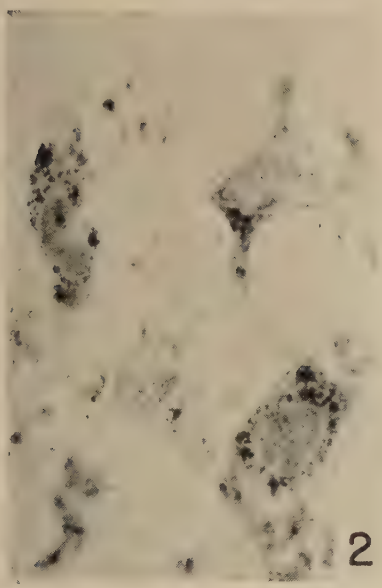
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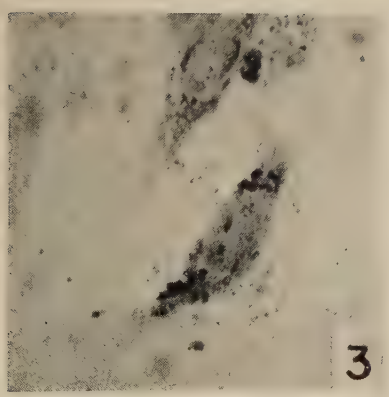
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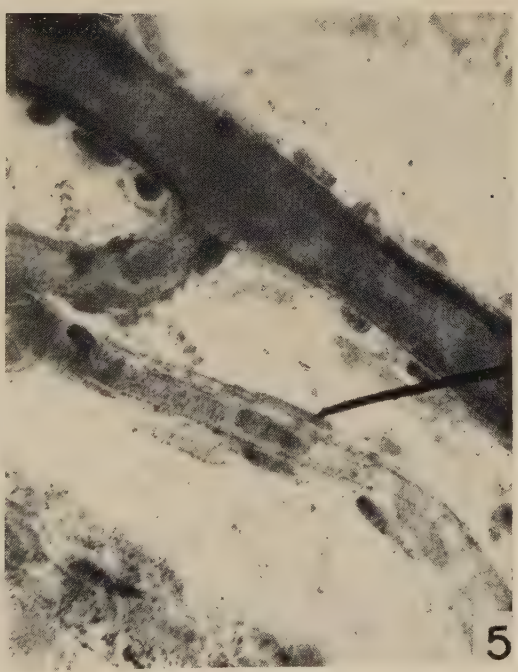
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HISTOGENESIS OF THE CELLULAR ELEMENTS IN THE POSTOTIC MEDULLA OF THE CHICK EMBRYO

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ELEVEN FIGURES

INTRODUCTION

Interest in the histogenesis of the cellular elements of the postotic region of the medulla grew out of several factors. Earlier a study of the differentiation of the nerve pattern of this area in chorioallantoic grafts was reported (Reddick, '45). This, however, contributed little to the understanding of the day by day differentiation of the individual cells. Most studies on the histogenesis of cells in the neural tube have been made from cross, longitudinal, or sagittal sections (Lenhossek, 1839; Hardesty, '04; Cajal, '09; Cowdry, '14; Sauer, '35; Barron, '46; and others). It is very difficult to determine the limits of individual cells from such sections. This is especially true of the cytoplasmic limits of cells that make up the early mantle layer, since the whole cell cannot be demonstrated in any one section with any degree of certainty For this reason the cellular processes of early neuroblasts and spongioblasts must be traced from one section to another, or only a section of the cell can be considered.

It was anticipated that by smearing the cells of this region, they could be separated. In so doing it would be possible to determine whether or not there was an anastomosis of the cytoplasm of the cells at any time during early development.

[1] Aided by Morehouse Graints-in-aid Committee in cooperation with the Carnegie Foundation.

The role of epithelial cells in their relation to "germinal cells" and differentiating spongioblasts and neuroblasts was considered.

Results indicate that each cell is an entity within itself, with its own cytoplasmic limits. Differentiation of the cytoplasm into processes of either neurons or neuroglia could be traced from the 2nd day of incubation through the 7th day. Toward the end of the 3rd day through the 7th day, the neuroglial fibers form a network in which their nuclei and the cell bodies of differentiating neurons become embedded. As early as the second day epithelial cells show differentiation toward neuroblasts or spongioblasts. Mitotic figures in the epithelial cells and "germinal cells" indicate that they are the same.

MATERIALS AND METHODS

White Rock embryos incubated from 2 to 7 days were used. Smears were made at 24 hour intervals. In cases reported below, each group of smears, from 2 to 7 days, was made from eggs which had been placed in the incubator at the same time.

The embryos were removed directly from the egg to a Petri dish filled with normal saline. The postotic medulla was dissected out of the embryo under a binocular stereoscopic microscope with iridectomy scissors and watch-maker's forceps. In older stages the peripheral nerves were removed with their accompanying ganglia. The medulla was cut just below the ear vesicle which marked the anterior limit of the part investigated. The posterior border of the 4th somite was used as the posterior limit of the medulla. The small piece of medulla was then transferred to a clean slide with a short, small-mouthed pipette, the bulb of which was controlled by squeezing it between the thumb and palm of the hand. In this way only a small amount of saline solution was transferred to the slide; by gentle suction with the same pipette, all excess solution was easily removed.

Thin or thick smears were made by controlling the amount of pressure placed on the slide used for spreading the cells. In many cases the cells were not smeared, but mashed by the top slide; this prevented distortion of the cells.

Most of the observations were made on cells fixed in 90 cm^3 of 70% alcohol, 5 cm^3 of formalin and 5 cm^3 of acetic acid and stained with protargol-S. The cells remained in the fixative from 2 to 4 hours, and were washed in 70% alcohol until the odors of acetic acid and formalin were gone. The general procedure suggested by Bodian ('36) was followed except that no copper was used. It was found that best results were obtained by letting the slides remain in the protargol at 37°C. for from 2–6 days depending upon the age of the cells and the thinness of the smear. Some smears were stained with a special grade of protargol, N-051, which Winthrop-Stearns could no longer prepare. With this stain copper was used in the way suggested by Bodian ('36). These will be discussed only in comparison with the smears stained as described above. The cells were studied and drawn under oil immersion.

OBSERVATIONS

Two-day embryos. The two-day smears were left in protargol for 5 or 6 days in order to get the stain to take effectively.

Three types of cells were identified according to the size and staining reactions of the nuclei. These were the "germinal cells," epithelial cells and early neuroblasts. The "germinal cells" are large rounded cells in which the nuclear membrane is broken down and the chromatin is in early stages of mitosis located near the central portion of the cell. The epithelial cells are of two types. In one type, which are believed to be early spongioblasts, the nuclei are small, round and heavily granular. In the other type of epithelial cells the nuclei are larger, oval-shaped and finely granular. These are believed to be early neuroblasts. In both types of cells the cytoplasm is typical of that of epithelial cells (figs. 1 b, d, e and 6).

Mitotic figures are present in both types of epithelial cells. Most of these figures are in the late anaphase stage. There

are cells (fig. 1 c) in anaphase that appear to be transitional between the two. This suggests that the "germinal cells" may be epithelial cells whose cytoplasm is withdrawn at the time of early mitosis and extended as the process is completed.

In certain smears, where there was little pressure applied, the epithelial cells were just slightly separated. The arrangement of such cells gives the impression that the end of the cytoplasmic process of a cell may grow in between those of two other cells pushing them apart and making a wedge for itself as it does so. This appears to occur at both ends of the cell (fig. 1 a). There is at no time any indication of anastomosis between these cells.

In several smears of two-day embryos, processes were seen which may have been early axons. These processes were studied carefully where seen in order to be sure that they did not result from smearing. When they appeared in a region where the cytoplasm around cells in the vicinity of them were in the epithelial character, they were considered as initial outgrowths. One such cell is shown (fig. 1 f) with what appears to be a growth cone at the end of it.

Three-day embryos. General appearance of the smears: Around 200 smears of three-day medullas were studied. About one-half of these were stained with protargol N-051, the others with protargol-S. The former stained only the nuclei and fibers from the neuroblasts and spongioblasts. They did not show the relationship of the fibers to the cells, which gave the impression that a large number of nuclei were suspended in a fibrous meshwork. However, even in these, the three types of nuclei as described in the two-day smears could be distinguished.

In smears stained with protargol-S the nuclei, cytoplasm and fiber relationships were clearly brought out. Smears of embryos of the same chronological age, and approximately in the same morphological stage, which are fixed and stained under similar conditions, give different over-all pictures. In practically all cases, however, one is surprised at the large

number of fibers present, and the progress in differentiation of the three-day medullas over those of the two-day embryos. At this stage the fibers of the spongioblasts and neuroblasts have neither migrated too far away from the cells, nor have been sheared away from the cells by smearing for the relationships to be seen. In some smears many of the cells are in stages of mitosis, and there are fewer fibers throughout the total smears.

As is known, there is a wide variation in the development of chick embryos, although they may come from the same batch, and are incubated under the same conditions. Two grafts typical of the ones indicated above will be described in detail.

P_3A_3. This was a smear of an embryo with 37 somites and is typical of an average three-day smear which shows more differentiation than mitosis. The three types of cells which were described under two-day smears are present in addition to cells which have differentiated as neuroblasts or spongioblasts.

Neuroblasts. Monopolar and bipolar neuroblasts are present. There are two types of monopolar and bipolar neuroblasts. In one type the nuclei are large and oval, while in the other of the two types, they are less oval (almost round) and smaller (figs. 2, 4 d, e, 5 c, d–l). The nucleoplasm is very finely granular in both types of nuclei. The monopolar neuroblasts show very little if any protoplasm around one pole of the nucleus, whereas the axons are long and very heavily stained. In very thin smears neurofibrillae can be seen near the proximal end of the process embedded in the protoplasm.

In the bipolar neuroblasts both processes begin at points about equidistant from the nucleus, but the axon is much longer than the dendritic outgrowth (figs. 2 and 7).

Spongioblasts. The spongioblast nuclei are rounder and more heavily granular than those of the neuroblasts. These cells differentiate in two directions: in one direction toward astroblasts and ependymal cells; in the other toward oligodendroblasts. In the first case the nucleus becomes smaller

and more heavily granulated. Finally the nucleus becomes very small and barely perceptible, leaving only what appears to be an irregularly shaped darkly-stained mass from which fibers radiate. These steps can be traced throughout the series from 3 days to 7 days with more of these differentiating in the older embryos (figs. 4, 5, 8, 10 and 11).

The nuclei of the oligodendroblasts are not as heavily granulated as those of the astroblasts, nor is there as much reduction in size. The cytoplasm of both types of cells undergoes similar differentiation up to a point. In transforming from an epithelial cell to a spongioblast, vacuolated areas appear in the cytoplasm some distance from the nucleus. Immediately around the nucleus the cytoplasm stains heavily. From this area many processes grow out in all direction. In areas where three or more of these cells are together there is an intermingling of their processes (fig. 7). Some of these processes extend some distance from the nucleus, and there are indications that they may eventually lose connections with the nucleus. Many such processes give the fibrous meshwork appearance to the smears.

P_3A_{11}. This is a smear of a three-day embryo with 34 somites. All of the cellular types described above are present, but fewer in number. Many of the cells are in mitotic stages (fig. 3). This condition was encountered quite often. Mitotic figures are also seen in cells which have differentiated into monopolar neuroblasts, but most of the cells showing mitosis are still in the initial epithelial condition.

Four and 5-day embryos. These will be considered together because there are only a few significant differences between these smears and those of the three-day embryos. The most noticeable difference is the increase in the number of cells differentiating as neurons or neuroglia.

Neuroblasts. There are more bipolar neuroblasts of both types (small and large nucleated) present. It is noticeable that differentiation of these cells appears in areas along the smear.

Monopolar neurons of both types are present in large numbers; some of these show indications of dendritic formation (fig. 8).

Spongioblasts. With differentiation of the spongioblasts into astrocytes and oligodendrocytes there is a change in the texture of their fibers. They take a deeper stain, and their more distal fibers form a very fine meshwork of material that gives the appearance in the smear of the typical white matter of the brain. This meshwork shows neuroblasts and spongioblasts within it. Gliosomes are present on some of the oligodendroblasts. A typical cell of this type is shown in figure 10. Astrocytes are shown in the same figure.

Beginning with the 4th day and progressing throughout the series, some of the oligodendroblasts and astroblasts differentiate in relation to the blood vessels. It is in these cells that gliosomes first appear in the oligodendroblasts.

Six-day embryos. All of the cell types discussed above are present, but there is a great increase in the total number of fibers from both supporting and conducting elements. One case will be discussed.

P_6A_6. Neuroblasts (neurons). Bipolar and monopolar neuroblasts and multipolar neurons are present. There are more of the first two types than the latter. The multipolar neurons show two or three dendrites and an axon. It appears that the dendrites result either from a withdrawal of the original first dendrite and a redistribution of the protoplasm, or a redistribution of all of the protoplasm of the cell. This hypothesis is drawn from the fact that the axons of the mutipolar cells are shorter than those of the bipolar type, and the dendrites of the multipolar neurons are shorter than the original long dendrite (figs. 4 and 5). The dendrites appear to grow out in all directions. By focusing up on some of the cells whose nuclei can barely be seen for the cytoplasm covering them, one or more processes can be seen on this surface of the cell in addition to those seen by focusing down. The nuclei of these cells are smaller than those of the large nu-

cleated bipolar types and contain one or two darkly stained nucleoli (figs. 4, 5 and 11).

Spongliоblasts (neuroglia). Definite neuroglial elements are present, many of which have reached their definitive condition. Spongioblasts which are differentiating as described above are present in large numbers. The oligodendroglial cells are larger in number; the nuclei are small and the cytoplasmic fibers show pronounced gliosomes. The astrocytes show small nuclei, or no nucleus with darkly stained fibers passing from them (figs. 4, 9, 10, 11).

Seven-day embryos. The overall picture of these smears is very much like that of the 6-day medulla. More multipolar neurons and astrocytes are present (fig. 5).

DISCUSSION

There has been much controversy concerning the origin of conducting and supporting elements of the nervous system in relation to the epithelial cells and "germinal cells." Discussions of these differences of opinion are summarized in the works of Cajal ('09), Penfield ('32), Kappers, Huber and Crosby ('36); and in introductory remarks on the nervous system in textbooks of histology and embryology (Bailey, '44; Maximow and Bloom, '48; Arey, '46; Patten, '29; and Lillie, '36).

The observations reported above indicate that the "germinal cells" are the same as the epithelial cells, and that the "germinal cells" are no more than epithelial cells passing through the early stages of mitosis. This is in agreement with conclusions drawn by Sauer ('35). The epithelial cells are able to differentiate as spongioblasts or neuroblasts. Indications of this differentiation occurs as early as the second day. These cells are continuously produced and some may remain undifferentiated as long as the 7th day, while others differentiate into specific conducting or supporting elements. This is in accord with the findings of many investigators: Schaper (1897); Cajal ('09); Brizbee ('49) and others.

Cells comparable to microglia were not identified in the smears, although they may have been present.

SUMMARY

1. Smears of the postotic medulla of embryos from 2 to 7 days old have been described.

2. There is a progressive differentiation of neuroblasts from the 2nd to the 7th day from epithelial cells through monopolar, bipolar and multipolar stages.

3. There is a similar progressive differentiation of spongioblasts from epithelial cells into astrocytes and oligodendrocytes.

4. During early development the neuroglial cells are distinct. Each has its own cytoplasmic limits. Later, as fibers are produced, they form a meshwork in which the nuclei of these cells appear to be enmeshed.

5. "Germinal cells" and epithelial cells are present in 7-day embryos, and are the source from which cells in early stages of differentiation are derived.

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PLATES

PLATE 1

EXPLANATION OF FIGURES

All figures are camera lucida drawings of protargol stained cells. × 1918.

1 P_2A_5. Drawings of types of cells in smears of two-day postotic medullas.

a. A group of epithelial cells showing distinct cytoplasmic membranes.

b. "Germinal cells."

c. A cell transforming from the rounded to the epithelial condition.

d. An epithelial cell differentiating toward spongioblast formation. Note the rounded granular nucleus.

e. An epithelial cell differentiating toward neuroblast formation. Note the larger, oval-shaped nucelus.

f. Early monopolar neuroblast.

2. P_3A_3. Drawing of photogram shown in figure 7.

a. Monopolar neuroblast — the axon extended beyond the area of the drawing.

b. Bipolar neuroblast with axon longer than the dendrite. The two fibers shown are from cells beyond the area drawn.

c. An early oligodendroblast.

d. "Germinal cells."

3. P_3A_{11}. A group of epithelial cells, all of which show mitotic figures.

4. This is a composite drawing to show features discussed above. The drawings are from smear P_6A_5.

All figures are camera lucida drawings. × 1918.

a. Oligodendroblast showing gliosomes.

b-1–b-4. Changes in the nucleus that occur as a spongioblast differentiates into an astrocyte.

c. A blood vessel showing astrocyte processes applied to it.

d. A monopolar neuroblast of the type with a large oval nucleus.

e. A monopolar neuroblast with a small oval nucleus.

f. A bipolar neuroblast in which axon and dendrite are approximately the same length.

g. "Germinal cell."

h. An epithelial cell differentiating toward neuroblast formation.

5 This is a composite drawing of a 7-day smear showing features discussed above.

a. "Germinal cell."

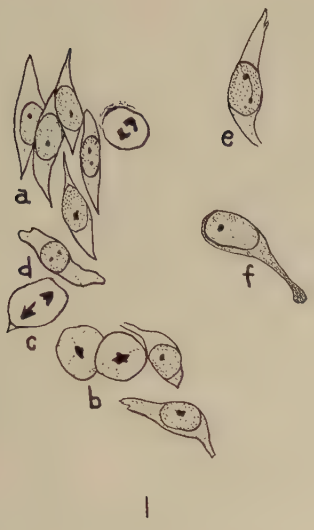
b-1–b-2. Epithelial cells differentiating toward spongioblast and neuroblast formation.

c. Monopolar neuroblast of type with small round nucleus.

d-1–d-2. Monopolar neuroblasts with the larger rounded nuclei.

e. Multipolar neuron.

f. Blood vessel showing mature astrocyte.



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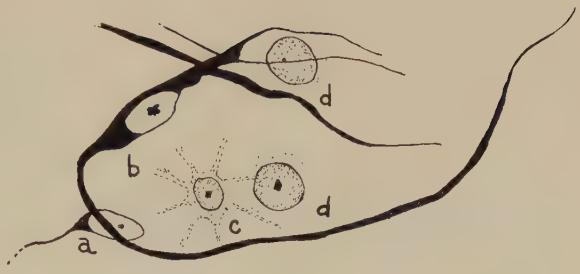

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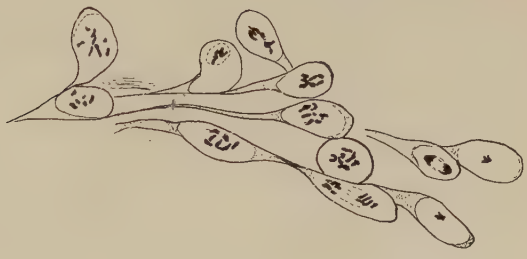

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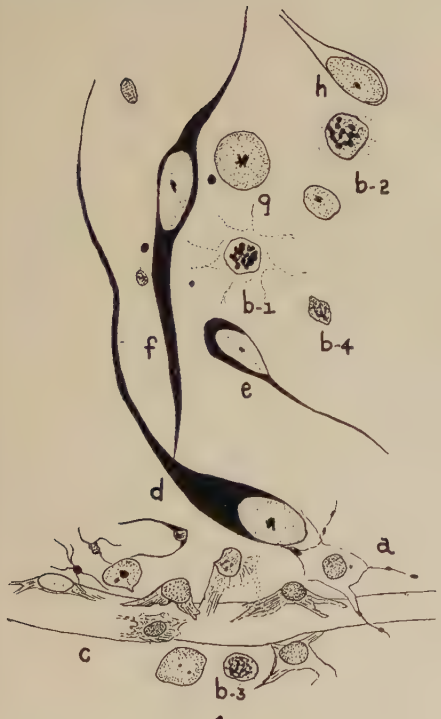

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PLATE 2

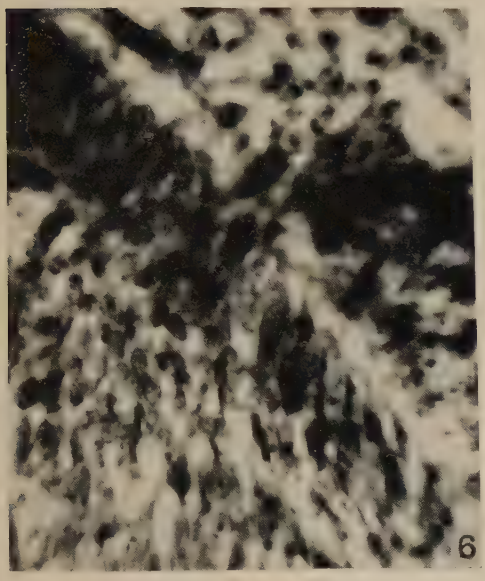
EXPLANATION OF FIGURES

These figures are photomicrographs.

6 P_2A_2. Smear of two-day postotic medulla. Note in thin part of smear epithelial cells with oval and rounded nuclei. Some cells show darkly stained processes (early neuroblasts); others in same region show cytoplasm of epithelial character. One large "germinal cell" may be seen near the center of the photograph. × 986.4.

7 P_3A_3. Photograph of smear from which drawing in figure 2 was taken Note that axon fades out in photograph since it runs deeper into the smear; at lower end of figure and between cell body and axon, oligodendroblasts are seen. × 986.4.

8 P_4A_6. Photograph of a 4-day smear. Monopolar and bipolar neuroblasts are seen in the center of the figure. Between and around these are spongioblasts in different stages of development (see fig. 4). × 986.4.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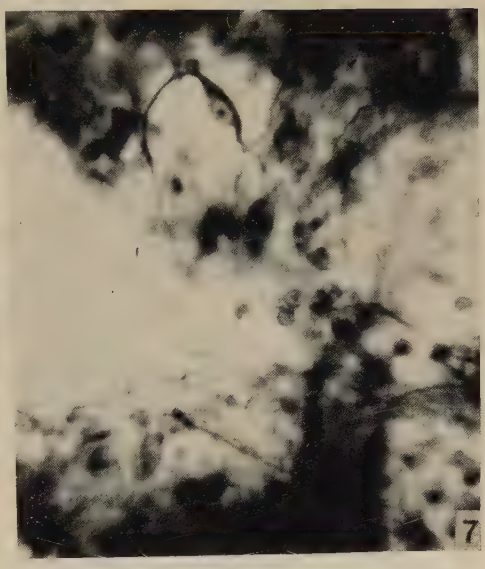
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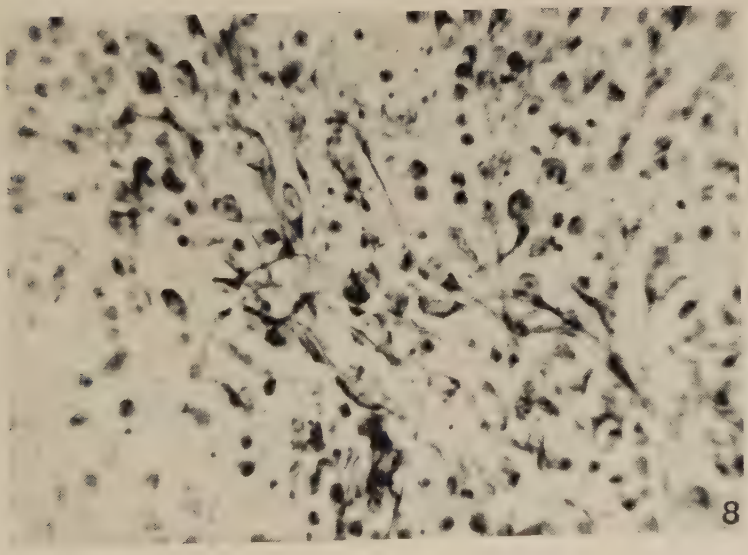
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PLATE 3

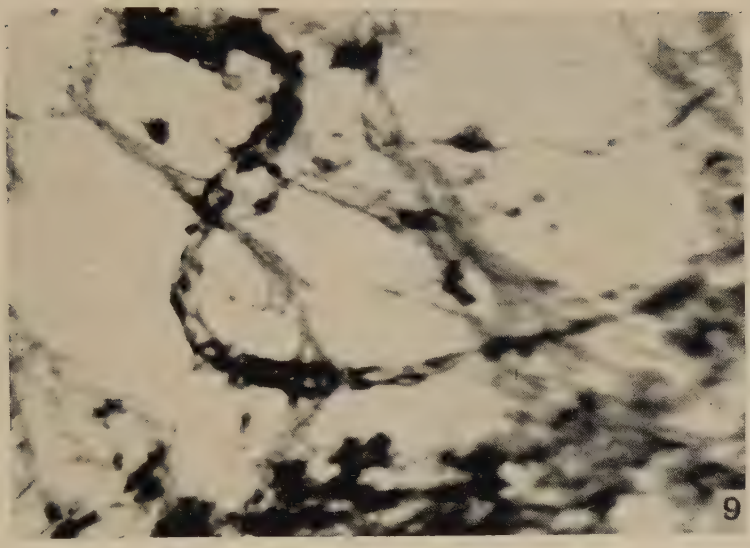
EXPLANATION OF FIGURES

These figures are photomicrographs.

9 P_6A_5. Photograph of a 6-day smear. Note increase in number of neuroglia fibers, and astrocytes moving toward and applied to blood vessel. × 986.4.

10 P_6A_6. As above, with oil immersion. Note gliosomes on the fibers of the oligodendrocyte. × 1.596.

11 P_6A_7. A 6-day smear. Note multipolar neuron, triangular-shaped cell in the center of the smear. Note small neuroglial cells, epithelial cells, etc. in smear. × 986.4.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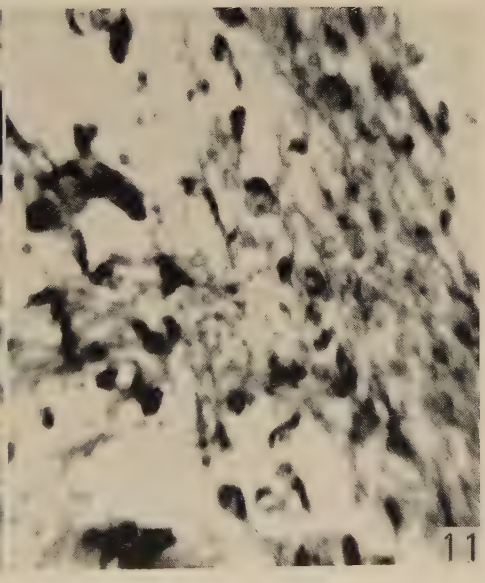
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INNERVATION OF THE PARS DISTALIS OF THE ADENOHYPOPHYSIS STUDIED BY PHASE MICROSCOPY

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ELEVEN FIGURES

INTRODUCTION

Whether or not the pars distalis of the hypophysis has a rich innervation has been in dispute for many years. Certain workers (e.g., Pines, '26; Brooks and Gersh, '38, '41; Vasquez-Lopez, '48) have claimed a rich innervation. Others (Croll, '28; Hair, '38; Rasmussen, '38; Green, '46, '47) have found only a few nerve fibers or even none at all. Many other workers have investigated this subject (for references see Green, '46; Harris, '48; and the papers cited) and the matter is still unsettled. Tello ('12) described connective tissue nets surrounding cells in the pars distalis. Pines ('26) and Brooks and Gersh ('38, '41) saw similar structures and interpreted them as nerve endings. The whole dispute has, in fact, hinged around the question of whether the strutures observed were nerve fibers or connective tissue fibers. Very few workers have gone into the subject of criteria. Green ('48) has, however, suggested certain standards by which a given fiber may be judged. It is not possible to prove that nerve fibers do not occur in the pars distalis. However, circumstantial evidence can be obtained and the present paper is concerned with this. It was considered likely that, if nerve fibers exist in the pars distalis, their physical and chemical properties should be similar there to comparable fibers in other parts of the pituitary. It was

[1] John and Mary Markle Scholar.

decided, therefore, to use phase microscopy in an endeavor to reveal them.

METHODS

A Cooke-Troughton-Simms phase microscope equipped with a monochromatic light source (mercury vapor lamp and Corning filters for the green band at 5461 Å) and 0.25 λ phase plates was used. A variety of mounting media of different refractive indices was employed ranging from ethyl alcohol (R_D 1.3624) to a saturated solution of mecuric iodide and potassium iodide in glycerol ($R_D > 1.7$). Rabbit and human hypophyses were used, formalin fixed and sectioned at 2–10 μ. A large number of substances were employed in an endeavor to bring out specific elements. Among these were: Phosphotungstic acid; phosphomolybdic acid; ammonium molybdate; osmium tetroxide; gold chloride; silver nitrate; cadmium chloride; cobalt nitrate; uranium nitrate; mecuric chloride; potassium iodide; iodine in a solution of potassium iodide; etc., in solutions of various strength. In addition sections were extracted with a variety of solvents and treated with a number of acids and bases in an attempt to digest away interfering substances.

RESULTS

Sections mounted in media of low refractive index with or without treatment with phosphotungstic acid (5% aqueous solution for 5 minutes) show many fibers in the adenohypophysis and neurohypophysis. Conditions are not satisfactory for photography because of the many superimposed interference patterns, but a fibrous structure can be clearly seen. This is rather more evident at lower magnifications. Phosphotungstic acid caused a marked increase in overall contrast (as did phosphomolybdic acid and to a less extent Lugol's iodine, gold chloride, and osmium tetroxide: other substances were less valuable). This was especially true of collagenous fibers around blood vessels and in the capsule of the gland. In figures 1 and 2 the appearances of the neural lobe may be compared (same section but different fields) and in

figures 3 and 4 the appearance of the pars distalis after similar treatment is visible. These sections were mounted in Shillaber's immersion oil (R_D stated by manufacturer to be 1.515). Collagenous fibers can be seen in both instances but they are darker after the phosphotungstic acid has been applied. These fibers remain visible even in media of high refractive index. Figure 8 shows fibers around a blood vessel in the pars distalis of the same section after phosphotungstic acid treatment mounted in a medium of R_D of about 1.68. Here the nuclei of the glandular cells are just visible but there is little to be seen other than the collagenous fibers. In the neural lobe under the same conditions only a few perivascular collagenous fibers and red corpuscles were clearly visible. Nuclei could just be detected. These fibers are therefore collagenous fibers and this is witnessed by the fact that similar inter- and pericellular fibers may be traced to the delicate collagenous capsule in the rabbit's pituitary (fig. 5). It can be seen that some of these fibers and some of the perivascular collagen(figs. 6 and 7) could be interpreted as nerve fibers or nerve plexuses by the unwary. In certain situations the fibers assume bizarre appearances (fig. 9) and in others they can be traced to what appears to be "pericellular" nets (figs. 10 and 11). The author doubts whether these structures are truly pericellular but they resemble rather closely the published accounts and may well have such relationships. They are certainly related to blood vessels. Even in media of $R_D > 1.7$ they remain visible.

DISCUSSION

While the present study must be considered a failure for exact optical differentiation of nerve fibers in either the neural lobe or adenohypophysis, it is probable that many of the fibers seen in media of low R_D are in fact nerve fibers. Raising the refractive index does not appear to diminish the number of fibers seen in the pars distalis but in media of high R_D the neural lobe appears almost transparent except for red

blood corpuscles, a few perivascular and capsular collagenous fibers and indistinct nuclei. It is well established that nerve fibers are present in the neural lobe. If the structures described in this paper, fibers and pericellular nets, were of nervous origin then they should not be visible in media of high refractive index. The fact that they are visible and that they so closely resemble the structures described in the past as nerve fibers and nerve terminations is regarded as evidence against the adenohypophysis having any large numbers of nerve fibers or nerve terminations in it, especially since the number of fibers in the sections of pars distalis do not appear to alter greatly with changes in refractive index. It is possible that phase microscopy used with control sections or areas may be of use in identifying the nature of fibers in other parts of the body.

SUMMARY

Phase microscopic studies provide circumstantial evidence against a copious innervation of the adenohypophysis in the rabbit and in man.

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PLATE 1

EXPLANATION OF FIGURES

1 Human neural lobe. × 900 with 0.25 λ phase plate and oil immersion lens. Mounted in Shillaber immersion oil R_D 1.515.

2 Same section after treatment with 5% phosphotungstic acid. (Different field.)

3 and 4 Pars distalis before and after treatment with phosphotungstic acid. Data as in 1 and 2.

5 Edge of rabbit pars distalis. Data as above. Note fibers passing to delicate capsule.

6 Plexus of fibers in rabbit pars distalis. Data as above.

7 Fiber in rabbit pars distalis resembling nerve ending. Data as above.

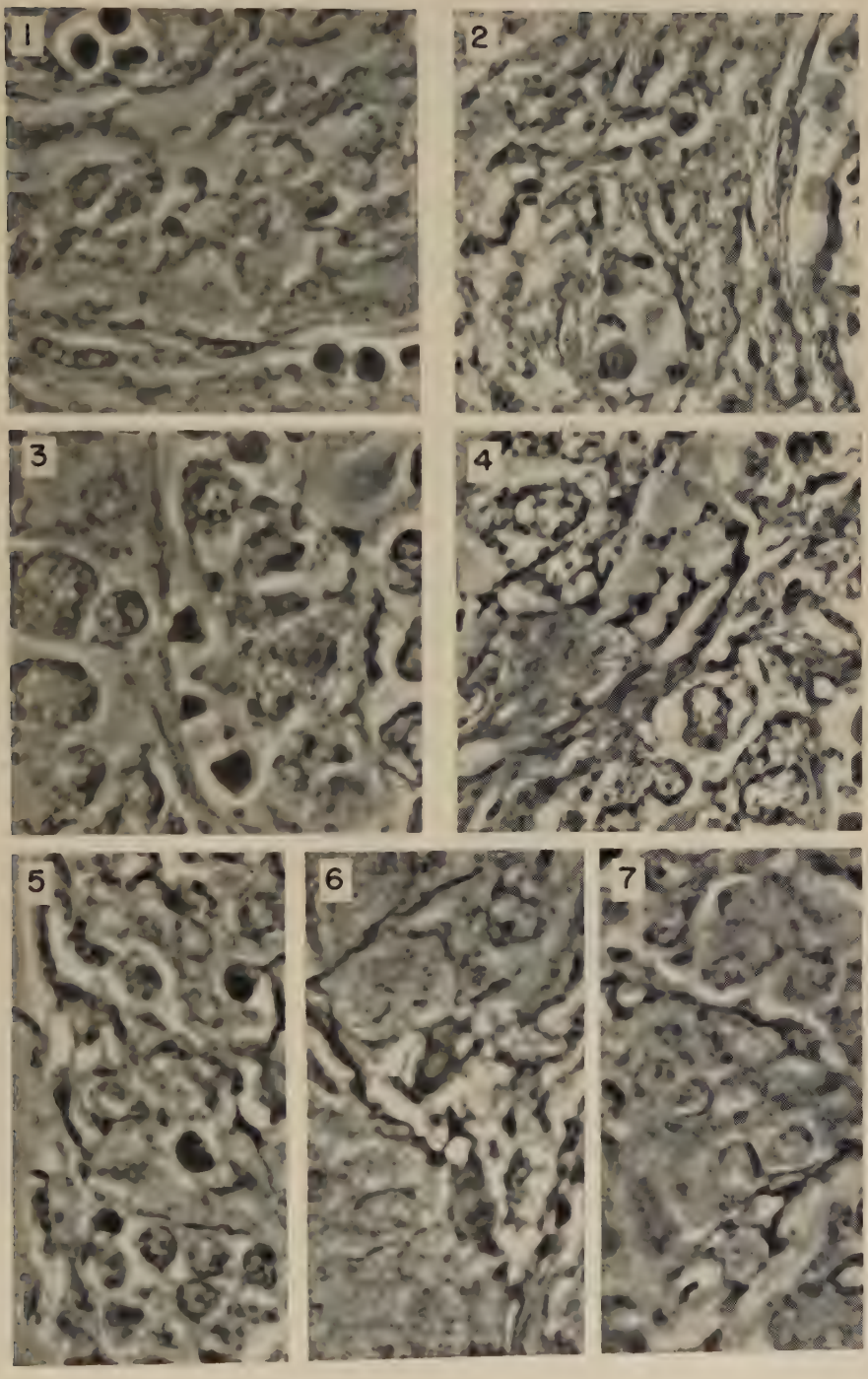
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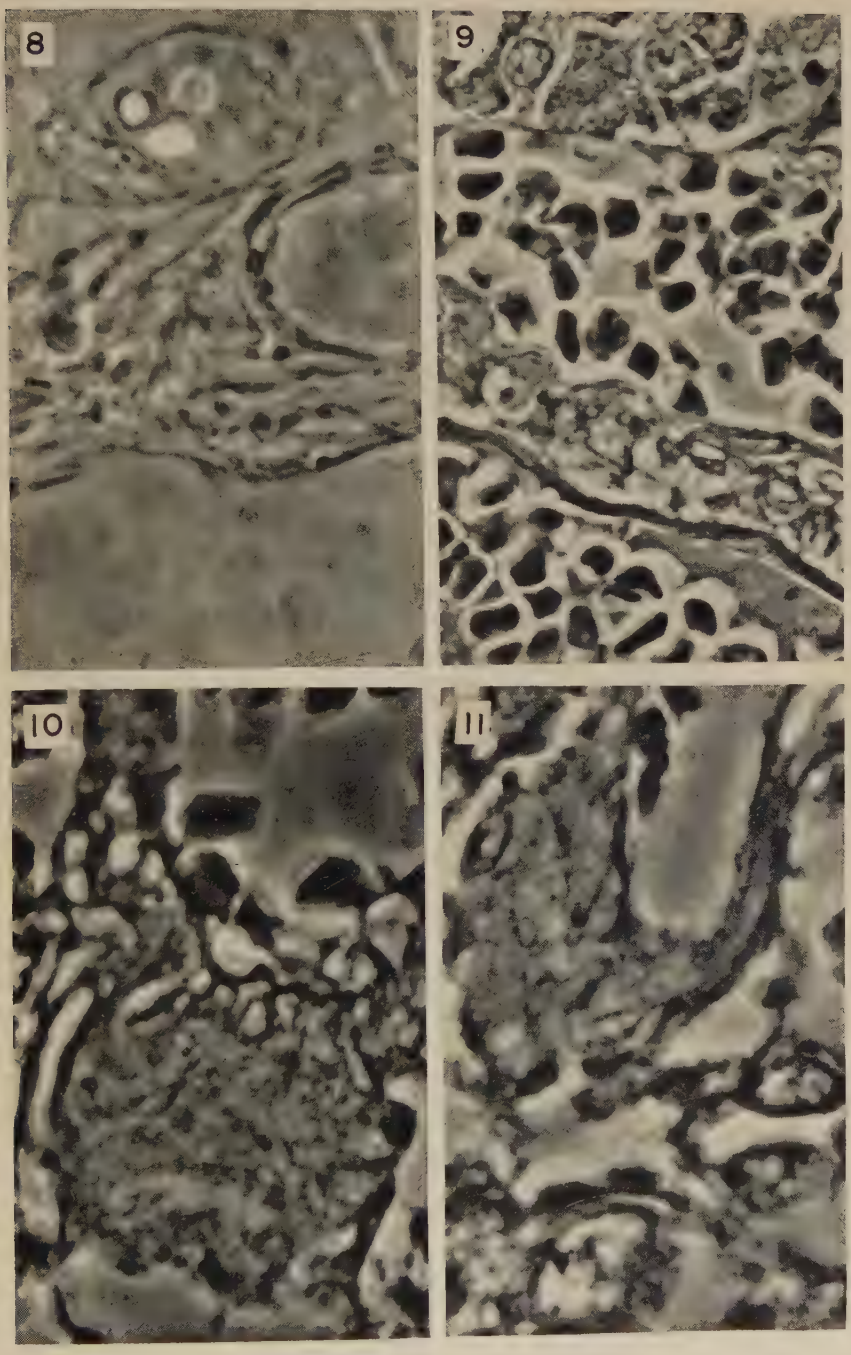
PLATE 2

EXPLANATION OF FIGURES

8 Human pars distalis. Data as in plate 1 but mounted in medium of R_D of about 1.68. Note suppression of detail in gland cells. Nuclei are just visible but no granules in cells.

9 Two hypophysio-portal vessels entering pars distalis. Data and mounting medium as in plate 1. Note reticulum between vessels and colloid in lower vessel.

10 and 11 "Pericellular nets." Magnification × 1500. Data otherwise as in plate 1.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AN IMPROVED METHOD FOR THE ARTIFICIAL INSEMINATION OF MICE[1]

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The purpose of this investigation was to determine a convenient and productive method for the large scale artificial insemination of mice. The success of artificial insemination is dependent both on the introduction of the sperm suspension at the proper time interval from ovulation and on the concomitant induction of the neurohumoral reaction necessary to elicit the progestational response of the uterus. Snell, Fekete and Hummel ('40) have shown the modal time for the onset of estrus in cyclic females to be from 4 to 6 hours following darkness. Ovulation occurred, on the average, from two to three hours following the onset of estrus. The onset of estrus was observed by them to be correlated with the diurnal cycle rather than with the specific hour of day. Mating the inseminated female with a vasectomized male has been used frequently as a reliable method for effecting implantation.

The techniques employed for the artificial insemination of mice have differed chiefly in one or both of two details, these being the estrus used and the avenue by which the spermatozoa were injected. The methods used by earlier investigators are given with those of the present work in table 1.

The techniques for the artificial insemination of mice at the postweaning estrus are described by Snell, Hummel and Abelmann ('43, '44). The approach of the first postweaning estrus was ascertained by them by examining daily the vaginal smears of females from which offspring about three weeks

[1] Work performed under Contract no. W-7405-Eng-26 for the Atomic Energy Commission.

old had just been removed. On the day the vaginal smear indicated estrus, the females were inseminated per vagina about 6½ hours following the onset of darkness. They were then permitted to mate with vasectomized males. Ninety-six vaginal plugs were observed following 153 inseminations. The sperm suspension leaked out following the insemination of 13 of the 96 females in which the plugs were later observed. Fifty-one of the inseminations proved fertile. Snell, Hummel and Abelmann ('44) also described a method whereby females

TABLE 1

	ESTRUS USED	AVENUE OF SPERM INJECTION	INVESTIGATOR
EARLIER WORK	Postweaning	vagina	Snell et al.
	Postweaning, following sterile mating	wall of uterine horn	Snell et al.
	Postpartum	ovarian capsule	Runner
	Cyclic	ovarian capsule	Runner
PRESENT WORK	Postpartum, 12 to 18 hours	vagina	Logan, Kile
	Postpartum, following sterile mating	vagina after plug removed	Kile
	Cyclic, following sterile mating	vagina after plug removed	Kile
	Cyclic, following sterile mating	wall of uterine horn	Kile

were inseminated by surgical procedure at the postweaning estrus immediately following mating with a vasectomized male. The sperm suspension was introduced directly into the uterus by an operative technique developed by Fekete and Little ('42). The fertility obtained by this method was "disappointingly low, varying from 10 to 44%."

It has been known for many years that, in mice, parturition is closely followed by ovulation. As noted in table 1, this postpartum ovulation has been used for artificial insemination

techniques. The recent work of Runner and Ladman (in press) has greatly clarified the confusing time relationship between parturition and the postpartum ovulation. They observed that the postpartum ovulation tends to take place nocturnally, irrespective of the time of day when delivery occurs. The interval between parturition and the postpartum ovulation varies between 12 and 18 hours, depending on the hour of delivery. Runner ('47) stated that litters were obtained from successful inseminations at the postpartum estrus. Since the time of ovulation at the postpartum estrus could be closely predicted according to Runner's data, we first attempted to inseminate females at this estrus. However, as will be shown, the results obtaind by such inseminations were quite unsatisfactory.

In an attempt to find a more productive method, our efforts were directed toward the cyclic estrus. The convenience of using cyclic females compared to postpartum ones can be readily appreciated, as the time consumed in the development of the first pregnancy is eliminated as well as the inconvenient waiting period of 12 to 18 hours between delivery and insemination. An additional three weeks for rearing the young were required when inseminations were made at the post-weaning estrus. Two techniques involving the use of cyclic mice were tried in our present work. We obtained good results with the second of these techniques: the injection of sperm suspension through the wall of each uterine horn following sterile mating.

METHODS AND RESULTS

Postpartum estrus. The work at the postpartum ovulation was done with females of two inbred strains. The animals were kept in a room in which the lights were operated automatically by an electric timing clock. The lights were turned off at 9 A.M. and on at 3 P.M. The cycle was thus controlled so that inseminations could be made during the normal working day. The time of delivery was observed or estimated, and the females were inseminated from 12 to 18 hours later at the

time predicted for the occurrence of ovulation. Approximately 0.1 cm^3 of the sperm suspension was introduced into each horn per vagina by a technique slightly modified from that of Snell, Hummel and Abelmann ('43). The vaginal speculum was made of glass and was held in a fixed position by a clamp on a ring stand. The shafts of the hypodermic needles used to inject the sperm suspension were bent 90° to give an unobstructed view of the os cervix with the aid of light reflected from an ophthalmoscope. Two 20-gauge needles with blunted points beveled in opposite directions were used, one for each horn. After insemination, females were placed with vasectomized males and kept under observation for the presence of plugs. Results obtained previously at this laboratory from

TABLE 2

Results from inseminating postpartum females

	LOGAN	KILE
Females inseminated	110	44
Plugs observed	73	35
Number of litters	22	9
Mean litter size	..	3.7 ± .56

similar work by R. E. Logan are given with the present results in table 2.

It seemed possible that inaccurate estimation of the ovulation time was responsible for the poor results obtaind. Our next approach, therefore, was to use the appearance of a vaginal plug from a natural mating as a convenient indicator of the onset of estrus from which the time of ovulation might be more accurately approximated. The necessity for observing the exact delivery time is then eliminated. Pregnant females of C57 brown strain were placed with vasectomized C57 black males prior to delivery. Mating occurred from 9 to 14 hours following delivery. From two to three hours after mating, the plug was removed from the vagina with a pair of small blunt

tweezers and the sperm suspension was introduced through the cervix, after which the plug was replaced. The sperm were collected by stripping the vasa deferentia of a freshly killed C57 brown male with watchmaker's forceps. (This combination of males was used throughout our work as the paternity of the offspring could then be easily determined by the disposition of pigment in the eyes at birth as well as by the coat color a few days later.) The results obtained were little better than by the other method. Of 34 females inseminated, nine delivered litters.

Cyclic estrus. Because of the advantages, already enumerated, of using the cyclic estrus, the plug indicator method of estimating the onset of estrus was next attempted on cyclic females. It was found that in cyclic females the plug was attached so firmly to the vaginal wall that it was extremely difficult to remove mechanically without extensive injury to the vaginal mucosa and the uterine ligaments. For this reason, the introduction of the sperm suspension directly into the uterus by operative procedure was tried.

C57 brown females were maintained in a room in which the daily light cycle was controlled by an electric timing switch. As a matter of convenience, the room was kept dark in the daytime, as the onset of estrus in cyclic females is known to occur nocturnally. The lights were turned off at 7 A.M. and on at 3 P.M. The females were kept with vasectomized males and examined regularly for plugs at 8:45 and 10:45 A.M. and at 12:45 P.M., and sometimes later if the behavior of the mice indicated the female was nearing estrus. The time of mating could be ascertained within two hours in this manner. In addition, a closer approximation of the mating time could be made from the consistency of the ejaculate and the appearance of the vagina. (Immediately following mating the seminal fluid is in a viscous state. The plug soon acquires a hyaline appearance and becomes increasingly opaque.) The females were inseminated at from 1½ to 4½ hours following mating. From one to three females were inseminated with the sperm from one male. For the first 59 inseminations, the sperm from each

male was suspended in from 0.25 cm^3 to 0.5 cm^3 of a 0.9% sodium chloride solution. For the remaining 44, a solution containing 0.9% sodium chloride, 0.042% potassium chloride and 0.72% glucose was used. Quantities of the suspension varying from 0.02 to 0.10 cm^3 were injected with a 28-gauge hypodermic needle into each of the uterine horns, which were alternately lifted from the abdominal cavity with a small glass hook following midventral laparotomy 1 cm anterior to the clitoris. The abdominal incision was closed with two layers of silk sutures. The surgical procedure was carried out under ether anesthesia.

Of 103 inseminations performed, 74 (71.8%) were fertile. Six of these females were unable to deliver their litters, possibly because of operative damage incurred at the time

TABLE 3

Insemination of cyclic mice following sterile matings

SPERM MEDIA	NO. INSEM.	NO. FERTILE	PER CENT FERTILE	NO. LITTERS	MEAN SIZE OF LITTERS DEL.
NaCl	59	42	71.2	38	6.34 ± .44
Salt-Dex.	44	32	72.7	29	5.48 ± .46
Totals	103	74	71.8	67	5.97 ± .32

of insemination. One other female which was observed to start delivery ate all her young prior to the next observation. The difference between columns 3 and 5 in table 3 is attributable to these 7 females. The mean litter size of the delivered young was 5.97. It is quite possible that some of the newborn were eaten prior to counting, as cannibalism was not uncommon in this group. As is shown in table 3, the results obtained with the two types of sperm media were not significantly different.

Many of the females were inseminated two or more times. One female, which was inseminated 5 times, delivered three litters following two failures. Two other females were successfully inseminated three times each. Complete successive insemination data are given in table 4.

TABLE 4

Successive inseminations

INSEMINATION PER FEMALE	FERTILE INSEMINATIONS PER FEMALE	NO. OF FEMALES	PER CENT FERTILE INSEMINATIONS
5	3	1	60
3	3	2	76.2
	2	5	
2	2	9	70.6
	1	6	
	0	2	
1	1	31	72.1
	0	12	
	Total	68	71.8

DISCUSSION

In the present work, border cases such as questionable mating times were not excluded from insemination. It is thus possible that the percentage of fertility can be increased somewhat if inseminations are made only under optimum conditions. However, the success obtained here may already approach that occurring from natural matings in this inbred strain.

The data presented by Snell et al. ('44) for the uterine wall technique are inadequate for comparison; but, from the percentages given, the results obtained at this laboratory appear much better. The exact cause of the differences in results is not readily apparent. The major differences in the techniques were the anesthesia used and the interval between the sterile mating and the sperm injection. (It is assumed that "just mated," in Snell's description, meant not longer than one hour.) The latter difference would be important only if sperm in artificial medium do not retain viability sufficiently long to effect insemination when injected at a considerable interval preceding ovulation. It is suggested, however, that the use of barbiturate anesthesia by Snell et al. was the cause of their failure to obtain good results with this method.

In comparing the results of Snell et al.'s methods (per vaginal insemination at the postweaning estrus, which in their experience was more successful) with the one developed in the present experiment, it is obvious that the latter is more productive when calculation of the percentage of success is based on the total number of inseminations performed (71.8% versus their 33%, $t = 6.0$). Snell et al., in presenting their data, indicate that the percentage of success obtained by them is 61.4, a figure not differing significantly from our 71.8% ($t = 1.5$). However, their figure is based only on the number of females retaining the sperm suspension and mating with vasectomized males, thus excluding 46% of the inseminations performed. These must be regarded as wasted effort in a consideration of the productivity of the method.

It is apparent that the time for performing successful artificial insemination can be approximated more accurately by observing the time of a sterile mating than from the examination of vaginal smears. In addition to avoiding the general fallibility of the smear technique, individual differences between animals are automatically allowed for, which is not the case when a mean value must be used for estimation of ovulation time.

SUMMARY

Several methods for the artificial insemination of mice were tested, of which the following proved to be most satisfactory and more successful than any reported by other workers. Females were inseminated during the cyclic estrus at from 1½ to 4½ hours following mating with a vasectomized male. After mid-ventral laparotomy, the sperm suspension was introduced through the wall of each uterine horn with a 28-gauge hypodermic needle. Of 103 inseminations made within the C57 brown strain, 74 (71.8%) were fertile. The mean litter size was 5.97.

ACKNOWLEDGMENT

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Thanks are given to Dr. Liane B. Russell for her assistance in reading the manuscript and making several valuable suggestions.

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THE POSITION OF THE INTERNAL MAXILLARY ARTERY AND ITS QUESTIONABLE RELATION TO THE CEPHALIC INDEX

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INTRODUCTION

Studies by Ewing ('47) on Maronites of Lebanon and by Ehrich and Coon ('48) on Albanians indicate that the occipital flattening, unintentional cranial deformation and consequent marked elevation of the cephalic index found in these people is the result of cradling practices. The inference is drawn that in the very young the head form is plastic and frequently subject to permanent alteration as the result of post-natal influences.

Recently, however, Lurje ('47) has reported an association between the cranial index and the course of the internal maxillary artery. In the dissection of 100 cadavers, Lurje found that among the 76 adults the internal maxillary artery lay lateral to the external pterygoid muscle in 13 of 42 sides (31.0%) of brachycephalic individuals, in 47 of 64 (73.4%) of mesocephalic individuals, and in 43 of 46 (93.5%) of dolichocephalic individuals. It may be calculated that, even under

[1] The assistance of Dr. Donald J. Gray and Dr. Donald Stilwell of the Department of Anatomy at Stanford University is gratefully acknowledged. They generously contributed the data from an examination of 34 cadavers to the authors for inclusion in this report.

The authors wish to thank Dr. Mildred Trotter of the Department of Anatomy of Washington University School of Medicine for her permission to carry out the investigations and for reviewing the manuscript; and Dr. L. R. Boling of the Dental School of Washington University.

the assumption of total interdependence of the right and left sides, this yields a χ^2 of 20.3 with two degrees of freedom, and the probability of the correlation being ascribable to chance alone is less than one in 10,000.

Although post-natal influences may alter the head form, these influences would not constitute an appreciable factor in altering the course of the internal maxillary artery. Padget ('48) concluded from a review of other studies and her own observations that by stage 6 (embryos approximately 20–24 mm) the internal maxillary branch of the external carotid artery has already established a connection with the lower division of the stapedial artery at the junction of its maxillary and mandibular branches, and that the internal maxillary branch develops an arterial loop around the mandibular nerve, followed by subsequent obliteration of the primary medial limb of the loop.

If it can be confirmed that the course of the internal maxillary artery is significantly correlated with the cephalic index, the following may be inferred: the two observations may in part reflect a constitutional complex which, considered from the evidence given by the cephalic index, is diversely represented in various races; and as evidenced by the course of the artery, is determined in the embryo and is not appreciably influenced by post-natal factors at all.

MATERIALS AND METHODS

In order to collect a series of observations in a diverse population, these studies were conducted in three laboratories in different parts of the country. The dissections of the pterygoid region were made by students but were examined by one of us in every instance. The head measurements were made by the authors.

Thirty-four cadavers were examined at Stanford University, California. Dr. Donald J. Gray and Dr. Donald Stilwell each measured the cranial length and breadth in each case. In every instance the measurements agreed within 1 mm. Except for one male of unstated race from Panama, all the cadavers

were of white individuals. Seven were females, the rest males. Place of birth was recorded and included: one unknown; 15 from various parts of the United States; one each from Mexico and Panama; 16 from Europe including two each from England and Denmark and one each from Sweden, Norway, Russia, Germany, Austria, Switzerland, France, Spain, and Italy, and one not stated.

At the Department of Anatomy of Washington University in St. Louis 56 heads were examined by two of us (D. O. and H. M.). Both took the cranial measurements and achieved agreement within 1 mm in every instance. The series consisted of 19 male and 7 female whites and 23 male and 7 female American Negroes.

At the Anatomy Department of Wayne University, 36 preparations on 19 cadavers were examined by one of us (G. L.). Most of the measurements were retaken independently on a different day with a different instrument. In these instances the agreement was always within 2 mm. Except for three male American Negroes and one female American Negro, the cadavers were of white males. The place of birth is not known, but to judge from the surnames, the backgrounds were diverse and included Slavic, German, English and Irish elements.

The whole series consists, therefore, of 107 adults examined bilaterally and two dissections of one side only. Maximum length and breadth of the skull were measured and the cranial index ($\frac{\text{breadth} \times 100}{\text{length}}$) was calculated.

Lurje ('47) has described the two main variations in position of the second part of the internal maxillary artery. He says: "In the first variation . . . the internal maxillary artery extends on the outside of the external pterygoid muscle directly to the posterior surface of the maxilla, the whole second part in adults lying on muscle fibers." This form is called *superficial*. "In the second variation," Lurje says, "the internal maxillary artery passes on the inner aspect of the external pterygoid muscle, the anterior part lying on the external surface of the base of the pterygoid process of the

sphenoid bone and then passing between the tops of the external pterygoid muscle, which begins here close to the maxilla." This form is referred to as *deep*. In a few instances neither of these descriptions applies for the artery *pierces* the external pterygoid muscle.

FINDINGS

There were no appreciable differences in techniques or findings among the three laboratories where this work was conducted, and the data from all three has therefore been pooled.

As may be seen from table 1, there are no statistically significant differences in the average cranial index of cadavers with internal maxillary arteries both superficial, one superficial and one deep, or both deep. This is true for all sub-groups although the standard error has been calculated only for sub-series of 13 or more individuals. Furthermore, no evidence of an association between these two characters is to be found for the total males or the total females or for the entire series taken together.

It may be argued that the series is too small to warrant considering such a negative conclusion. It should be pointed out, however, that the series is somewhat larger than that on which Lurje came to other conclusions. Furthermore, it may be noted that even the two sub-series, of 13 and 26 individuals respectively, demonstrate a statistically significant difference between the mean cranial indices of American Negroes and whites when both internal maxillary arteries are superficial.

One may follow the method of analysis used by Lurje and divide the cranial index into three major divisions. As shown in table 2 the superficial and deep courses of the artery are not significantly different in respect to the form of the cranium in this series. The χ^2 value for two degrees of freedom is 0.3 and the probability of such a distribution being due to chance alone, even on the assumption of independence of right and left sides, is greater than 80%.

TABLE 1

Mean cranial index and course of the internal maxillary arteries in individuals

	RELATIONSHIP OF INTERNAL MAXILLARY A. TO EXTERNAL PTERYGOID M.												
	Superficial-Superficial			Superficial-Deep			Deep-Deep			Deep-Pierce		Pierce-Pierce	
	No.	Mean	s.e.	No.	Mean	s.e.	No.	Mean	s.e.	No.	Mean	No.	Mean
White males	26	79.2	0.8	14	78.1*	1.3	19	78.8	1.1				
American Negro males	13	76.7	0.8	5	75.0		6	77.8				1	73.4
White females	5	77.9		1	80.4		6	80.1		1	81.9	1	79.4
American Negro females	5	77.9		2	79.1		1	79.2					
Total males	40**	78.4	0.6	19	77.3*	1.0	25	78.6	0.9			1	73.4
Total females	10	77.9		3	79.5		7	80.0		1	81.9	1	79.4
Total both sexes	50	78.3	0.5	22	77.6*	0.9	32	78.9	0.7	1	81.9	2	76.4

* One individual is included in whom an occipital exostosis may have modified the cranial form somewhat (c.i. = 71.5).
** One individual is included of unknown "race."

TABLE 2

Relationship of the course of the external maxillary artery to the head form

	TOTAL	DOLICHOCRANIC c.i. = less than 75				MESOCRANIC c.i. = 75 to 80				BRACHYCRANIC c.i. = 80 or over			
	No.*	No.*	%	n*	s.e.	No.*	%	n*	s.e.	No.*	%	n*	s.e.
Artery superficial	123	23	18.7	14	4.6	64	52.0	37	5.8	36	29.3	22	5.3
Artery deep	88	15	17.0	10	5.0	44	50.0	27	6.7	29	33.0	19	6.3
Artery pierces	5	2	40.0			2	40.0			1	20.0		

* *No.* represents the number of dissections; *n* used in calculating the standard error is the number of individuals showing one or two sides of the corresponding type and it is a better and more conservative estimate of the number of independent occurrences.

DISCUSSION

It does not necessarily follow from the foregoing that there are no racial differences in the frequency of the different types of course taken by the internal maxillary artery. Indeed, in the present series there is a tendency for the deep position to occur less frequently in American Negroes (21 of 67 sides = 31.3 ± 7.9%) than in whites (67 of 147 sides = 45.6 ± 9.8%). The standard error of the difference — calculated on the basis of the number of individuals rather than on the basis of the number of sides — does not indicate a statistically significant difference at the 5% level although the probability of a chance difference of this extent is less than 10%. The frequency of the artery lying lateral to the external pterygoid muscle in the whites is 52.4% of 147 sides which compares closely to the figure of 54.3% of 447 observations reported in a compilation from institutions in England, Scotland and Ireland by Thomson (1891)[2] and is significantly but not greatly different from the figure reported by Lurje ('47) of 67.5% of 200 dissections. Lauber ('01) of Vienna reported only 17 (8.5%) of 200 sides to show the superficial form of the artery. In only 5 of the skulls was the superficial position found to be bilateral. Adachi ('28) describes Lauber's findings as "kaum glaublich."

Adachi ('28) has reported findings in the Japanese. He notes that the artery occasionally pierces the external pterygoid muscle, but in these rare instances he ascribes the artery to either the superficial or the deep category according to the amount of muscle which overlies the artery. Adachi found the internal maxillary artery lateral to the external pterygoid muscle in 237 of 256 (92.6%) sides in males and in 73 of 75 (97.3%) sides in females. For the total group of 331 dissections on 175 individuals Adachi therefore found 310 (93.7 ±

[2] It should be noted that by error both Adachi's ('28) and Loth's ('31) compilations for Europeans twice include the 93 dissections studied by Long (in Thomson, 1891). The data presented here from Thomson include Long's figures among others.

1.8%) to be superficial and the remainder deep.[3] This figure is significantly higher than that reported for any group of Caucasoids or American Negroes.

From the study cited above and from other relationships of the artery, Adachi ('28) concludes that the internal maxillary artery is a "racial blood vessel." The present study suggests the possibility of a somewhat different frequency of the two major types of the course of the second part of the internal maxillary artery in American Negroes and whites. It must be understood, however, that the same varieties of the course of the artery are found in whites, Negroes and Orientals, albeit in somewhat different frequencies. Furthermore, this conclusion does not imply the correlation with the cephalic index.

Failure to find such a correlation deprives those who would emphasize the congenital determination of the cephalic index, of the indirect evidence suggested by a relationship to the prenatal process of determination of the course of the internal maxillary artery. The present evidence does not affect the hypothesis of a considerable plasticity of the skull form.

SUMMARY

A study of 216 dissections of the pterygoid region on 109 cadavers reveals no correlation between the cranial index and the position of the internal maxillary artery with respect to the external pterygoid muscle.

This is at variance with the work of Lurje, who reported that the second portion of the internal maxillary artery usually lies deep to the external pterygoid muscle in brachycephalic individuals, and in dolichocephalic individuals it occurs more frequently superficial to that muscle.

The frequency with which the artery lies deep to the external pterygoid muscle is somewhat greater in whites (46% of 147

[3] The recalculated standard error of 1.8% is used instead of the original figure of 1.34% given by Adachi ('28) because the high correlation between right and left sides makes it more reasonable to base this statistic on the number of individuals examined (n = 175) rather than on the number of sides (n = 331).

sides) than in American Negroes (31% of 67 sides). Considered in conjunction with the still lower frequency previously reported for Japanese, this suggests that despite the lack of correlation with the cephalic index in this series, there may well be racial differences in the frequency of the different types of position of the internal maxillary artery.

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BOOK REVIEW

ATLAS OF HUMAN ANATOMY, DESCRIPTIVE AND REGIONAL. VOLUME II, SPLANCHNOLOGY, ANGEIOLOGY, NERVOUS SYSTEM, ORGANS OF SPECIAL SENSE. By M. W. WOERDEMAN, Professor of Anatomy, University of Amsterdam. 1950. 642 figures, 12 in color, + index; pages not numbered. $10.00; volumes 1 and 2, set, $18.00. The Blakiston Company, Philadelphia.

This is the second volume of a proposed three-volume atlas of the human body. Volume I comprises illustrations of the bones, joints and muscles. Volume III will deal with the peripheral nerves and blood vessels and with regional anatomy. Volume II contains 145 figures for the digestive system (including the teeth and gustatory organs), 57 figures for the respiratory system (including the olfactory organs), and 79 figures for the urogenital system. The cardiovascular system has 77 figures and the lymphatic system 25 figures. There are 99 figures for the central nervous system and 85 for the peripheral and 61 figures for the eye and ear. The section on the skin contains 13 figures. As in Volume I each drawing is of a specific dissection, rather than a composite of several dissections. The B.N.A. nomenclature is followed throughout, with an explanation in English under each title. The colored plates, of various viscera, are exceptionally good; more of them could be used to advantage.

As the author states in the preface, the atlas is fit for use by students of anatomy in dental schools. For this purpose 50 figures are devoted to the portrayal of the permanent and deciduous teeth, their occlusions and ways of eruption. Each drawing is thoroughly labelled.

In the section on the respiratory system the lungs are particularly well illustrated. There are shown, in addition to the usual figures, enlarged dissections of the bronchial trees and pulmonary arteries. Several excellent bronchograms are reproduced and clearly explained. For the diagrams of the broncho-pulmonary segments the author uses the accepted terminology and also includes that recommended by the international committee to consider bronchial nomenclature.

The courses of the peripheral arteries and veins are portrayed to a great extent by diagrams superimposed upon the bony structures. While this method shows the plan of the vessels to advantage, it does

not give the dissector a picture of their relationships. It is to be hoped that such pictures will be included in the section on regional anatomy in Volume III. As original dissections of the lymphatic vessels and nodes were not available, the author has projected his diagrams "after careful study and in accordance with data found in other atlases, textbooks, and in scientific literature." The illustrations are clear cut and well labelled.

The nervous system is dealt with in a thorough and comprehensive manner. Figures showing the gross topography are accompanied by enlarged dissections of internal structures and by frontal and horizontal sections. The nervous pathways of the brain and cord are indicated diagrammatically in enlarged sections, most of them modified from other sources. As in the case of the blood vessels, the branches of the peripheral nerves are shown projected upon the skin or bony framework, with few indications of their relationships. The courses of the cranial nerves are also pictured diagrammatically. At the same time the nuclei of origin and central connections of each cranial nerve are figured in detail. This grouping of central and peripheral branches should prove to be a great help to busy students. There are only 8 illustrations of the autonomic nervous system, 6 of them by other authors.

The book contains many reproductions of frontal, horizontal, and median sections, strategically placed among the other figures. There are also illustrations made with the aid of low power magnification, showing the mucous membranes of the mouth and digestive organs, the internal portions of the eye and ear, and the structure of the skin. Nearly 50 figures are used to portray various stages in the development of the fetus and infant. These figures are grouped with those of the same structures in the adult. Such an arrangement is designed to give the student a better understanding of the normal structure of the adult body and an explanation of most of its anomalies.

The index of 38 pages is quite adequate. This book has the same high quality of printing and binding as the first volume. The format of the two volumes has not been affected by the change of publishers.

Considering the two volumes which are now available, this is an excellent atlas of the human body. For a first edition there are few typographical errors or discrepancies in the assembly of the material. These volumes alone contain more illustrations than are found in other atlases. According to the author's statement, the third volume will contain the figures necessary to make complete descriptions of every aspect of the body. The result should be a truly monumental work.

R. A. Miller

ERRATUM

The Anatomical Record, vol. 108, no. 2, October 1950.

R. O'Rahilly, H. Debson and T. Summerfield King

Illustrations as printed, figure 2(a), page 230 and figure 3(a), page 232, together with their key diagrams on pages 231 and 233, are incorrectly transposed. The illustrations appearing on pages 230 and 231 should be figures 3(a) and 3(b) respectively; those appearing on pages 232 and 233 should be figures 2(a) and 2(b) respectively. The legends for these figures are correctly reprinted below in the order they should appear under the illustrations as they were printed on pages 230–233.

(legend for halftone illustration as printed on page 230)

Fig. 3(a) Photograph of anterior aspect. The cut end of the aortic arch has been retracted by forceps to the left hand side.

(legend for line engraving as printed on page 231)

Fig. 3 (b) Key diagram to figure 3 (a).

(legend for halftone illustration as printed on page 232)

Fig. 2(a) Photograph of left aspect viewed from below and behind. The superior intercostal artery is held up by a pin.

(legend for line engraving as printed on page 233)

Fig. 2 (b) Key diagram to figure 2 (a).

The legends are arranged for cutting and pasting under the proper illustrations.